user manual
proteomics

Ettan *MALDI-ToF*

User Manual

um 18-1144-01

Amersham Biosciences

Important user information

All users must read this entire manual to fully understand the safe use of Ettan™*MALDI-ToF*.

WARNING!

The Warning sign highlights an instruction that must be strictly followed in order to avoid personal injury. Be sure not to proceed until the instructions are clearly understood and all stated conditions are met.

WARNING! High Voltage

The High Voltage sign highlights an instruction that must be strictly followed in order to avoid contact with lethal high voltage above 1 kV. Be sure not to proceed until the instructions are clearly understood and all stated conditions are met.

WARNING! Laser Radiation

The laser radiation sign highlights an instruction that must be strictly followed in order to avoid exposure to hazardous laser radiation. Be sure not to proceed until the instructions are clearly understood and all stated conditions are met.

Caution!

The Caution sign is used to call attention to instructions or conditions that must be followed to avoid damage to the product or other equipment. Be sure not to proceed until the instructions are clearly understood and all stated conditions are met.

Declaration of conformity

Safety standards

This product meets the requirements of the Low Voltage Directive 72/23/EEC through the harmonized standard EN 61 010-1:1993 + A2:1995.

EMC standards

This product meets the requirements of the EMC Directive 89/336/EEC through the harmonized standard EN 61 326-1:1997 + A1:1998.

WARNING!

This is a Class A product. In a domestic environment, this product may cause radio interference, in which case the user may be required to take adequate measures.

The **CE** symbol and corresponding declaration of conformity is valid for the instrument when it is:

- used as a stand-alone unit, or
- connected to other CE-marked Amersham Biosciences instruments, or
- connected to other products recommended or described in this manual, and
- used in the same state as it was delivered from Amersham Biosciences except for alterations described in this manual.

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1 Introduction

1.1 The Ettan MALDI-ToF system

Ettan MALDI-ToF combines the protein knowledge of Amersham Biosciences with that of experts in protein identification (ProteoMetrics LLC) and mass spectrometry (Scientific Analysis Instruments Ltd.) to offer a MALDI-TOF mass spectrometer for fast, reliable protein identification.

The Ettan MALDI-ToF system consists of the instrument, the connected computer and the Ettan MALDI Software that controls the procedures and evaluations of results.

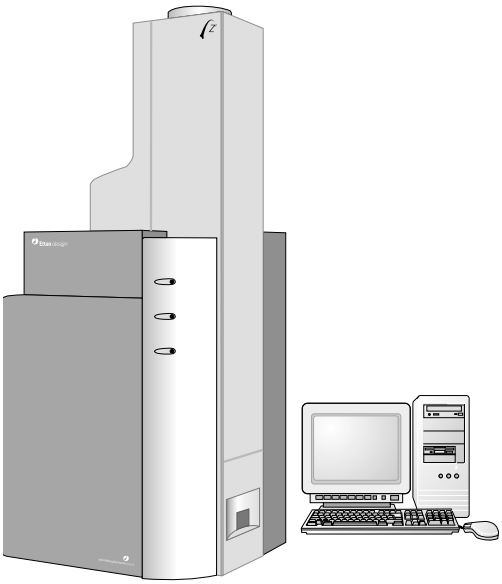


Fig 1-1. The Ettan MALDI-ToF system.

1.2 The User Manual

The Ettan MALDI-ToF User Manual provides instructions for performing MALDI-TOF MS analysis using the Ettan MALDI Software. Examples of sample preparation are described in Chapter 4 and practical examples of mass spectrometry studies are presented in Chapter 10. More detailed information on sample preparation and applications are found in the *Ettan MALDI-ToF Method Handbook*. For information on the instrument, the user is referred to the *Ettan*

MALDI-ToF Instrument Handbook. Detailed descriptions of all software menus and dialogues are provided in the *Ettan MALDI ToF MS Software Online Help*.

1.3 Principle of Ettan MALDI-ToF

A time-of-flight mass spectrometer separates ions of different masses and produces electrical signals that reflect the amount of ions at each mass. A simplified diagram of the time-of-flight principle is shown in Fig. 1-2.

In the Ettan MALDI-ToF instrument, a laser pulse desorbs and ionizes the biomolecules incorporated in a matrix on the sample slide. The ions are accelerated by high voltage, acquiring a velocity that depends on their mass: light ions acquire a higher speed than heavy ions. Entering a field-free "drift region", each ion keeps the constant velocity it acquired by the acceleration until it hits the detector. Ions of different mass can thus be separated in time: heavy ions take longer than light ions. The detector produces signals representing the number of ions hitting it at a certain instant.

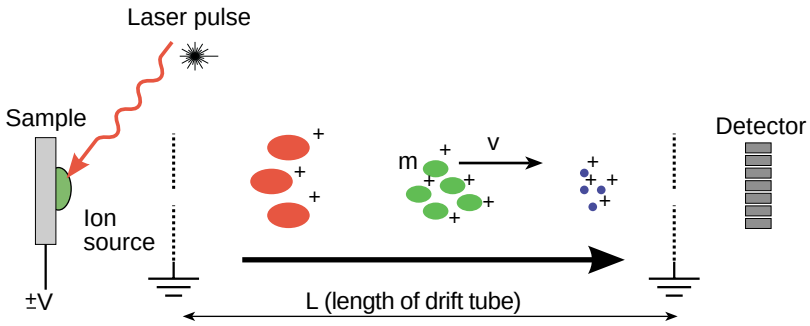


Fig 1-2. Principle of MALDI-TOF MS.

Since the time-of-flight (t) is proportional to mass over charge (m/z),

$$t = k\sqrt{\frac{m}{z}}$$

the detector signals are presented as a mass spectrum, a diagram where the signal intensity is plotted against mass over charge (m/z), which in MALDI usually corresponds to mass.

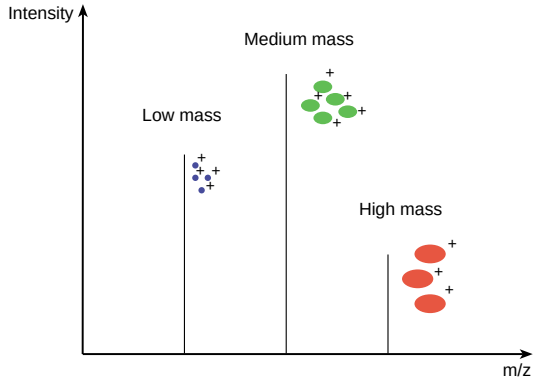


Fig 1-3. Example of a mass spectrum.

This simple principle corresponds to the linear mode in Ettan MALDI-ToF. This mode is convenient when analysing full length proteins (Mw >4000 Da). A higher sensitivity is obtained due to a higher transmission and detection of neutral products. However, for digest fragments, the use of a reflectron is preferable since it provides higher resolution.

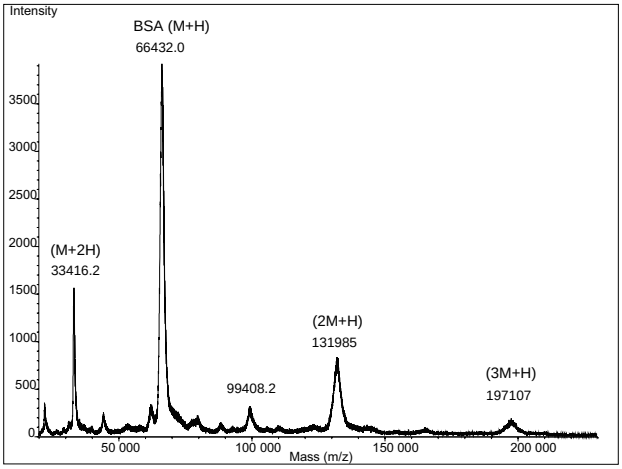


Fig 1-4. Mass spectrum of bovine serum albumin

1.4 The reflectron

The Ettan MALDI-ToF includes a harmonic reflectron that acts as an ion mirror (see Fig. 1-5). The reflectron improves resolution by extending the ion flight path. In addition, ions of equal mass having slightly different energy will be focused in time, thus giving more narrow isotopic peaks (higher resolution).

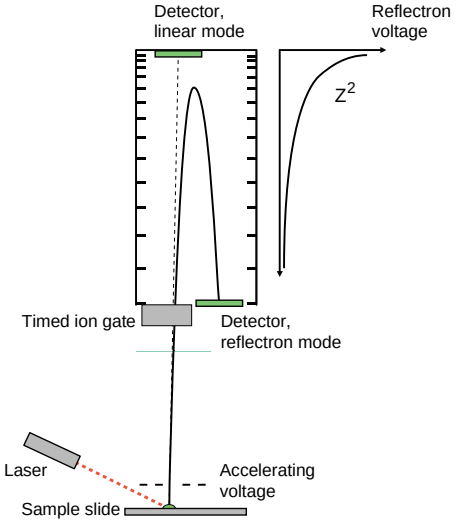


Fig 1-5. Harmonic reflectron principle.

1.5 Timed ion gate and PSD

The Ettan MALDI-ToF is equipped with a timed ion gate, which is used when analysing fragmentation caused by post-source decay (PSD). The m/z for a selected peptide ion (precursor ion) can be defined and only this ion and its fragment ions will pass the gate. The fragment ions can then be analysed to obtain sequence information.

1.6 Opportunities for protein and peptide analysis

Data from the Ettan MALDI-ToF will provide information for the following investigations:

- Determination of the molecular weight of a protein/peptide
- Identification of a protein using digest peptide masses and database searches

- Identification of a protein or characterization of a peptide, using PSD fragment masses and database searches

Three types of Acquisition method templates are available in the Ettan MALDI Software:

- Full length protein
- Protein digest
- PSD

Created Acquisition methods, together with created analysis methods in the Ettan MALDI Software, will support these laboratory protocols:

- Full-length protein analysis
- Peptide mass fingerprinting
- Protein identification using fragments obtained after PSD

1.6.1 Full-length protein analysis

The starting material is a purified protein, lyophilized or in solution. The protein is directly analysed by Ettan MALDI-ToF in linear mode (Acquisition method template: Full length protein). The result will usually consist of one or two peaks for each protein, since the ions normally have one or two charges. In addition, for some proteins, aggregates may be detected (see Fig. 1-4). This application is useful for determining molecular weight and as a purity control for a sample.

1.6.2 Peptide mass fingerprinting

The unknown protein is digested in-gel with e.g. trypsin. The proteolytic peptides (digest) are extracted from the gel and analysed by Ettan MALDI-ToF in reflectron mode (Acquisition method template: Protein digest). Peaks for the obtained peptides will be shown. The protein is identified by comparing the digest peaks with a computer-generated database of tryptic peptides from known proteins.

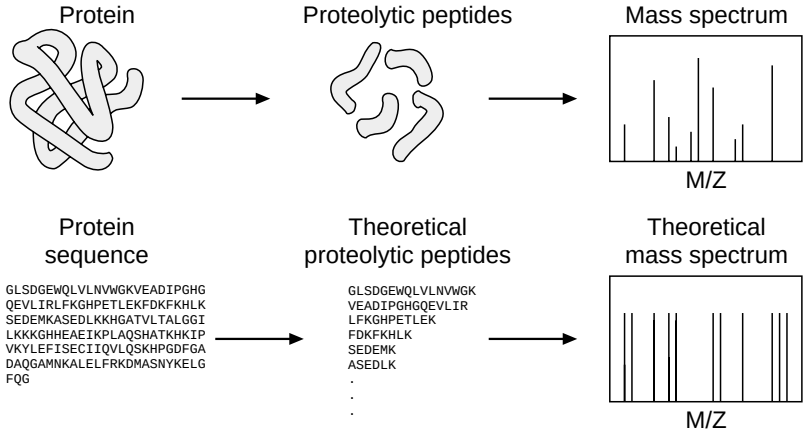


Fig 1-6. Protein identification by peptide mass fingerprinting.

1.6.3 Protein identification using fragments obtained after PSD

If identification of a protein failed when peptide mass fingerprinting was used, it is possible to obtain additional information by using PSD investigations. One of the obtained peptide masses is selected with the timed ion gate, laser power is adjusted to induce post-source decay and the obtained fragment pattern is studied. The recommended Acquisition method template in this case is PSD.

Example of a simplified description of PSD

A peptide of 15 amino acids is selected. The peptide has been ionized and the positive charge has been randomly situated at three different positions:

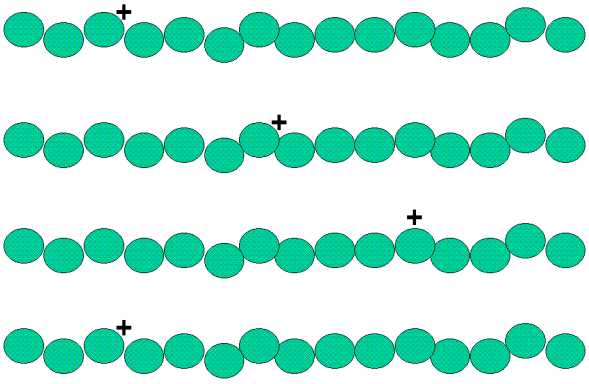


Fig 1-7. An ionized peptide.

The positive charges, together with the increased applied laser power, lead to excess internal energy at the bonds, which then break producing fragment ions and neutral fragments.

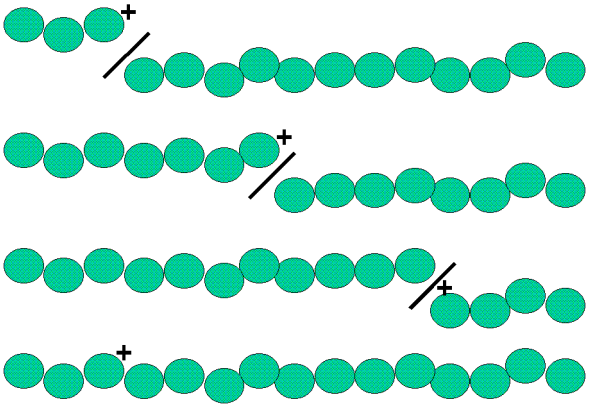


Fig 1-8. Cleavage of peptide bonds and production of PSD fragments.

The fragmentation occurs after the peptide is accelerated. Thus, all fragments will have the same velocity in the first drift region. It is therefore possible to use the timed ion gate for the analysis of the selected peptide and its fragments.

When the fragments arrive at the reflectron, the neutral fragments continue moving forward. However, the charged fragment ions are retarded and turn back towards the reflectron mode detector.

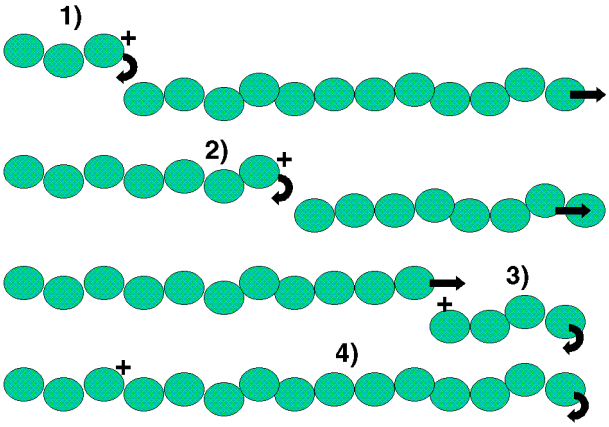


Fig 1-9. Fragment ions and neutral fragments in the reflectron.

The fragment ions and the precursor ion will be separated in the reflectron and four peaks will be obtained in the spectrum.

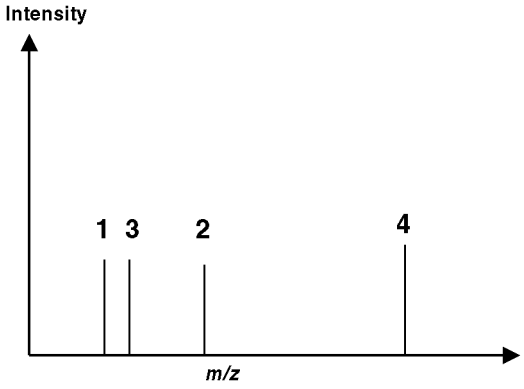


Fig 1-10. PSD fragment pattern.

There is a mass spectrometric fragmentation nomenclature, see Fig. 1-11. In MALDI-TOF MS analysis, fragmentation occurs usually at position B or Y.

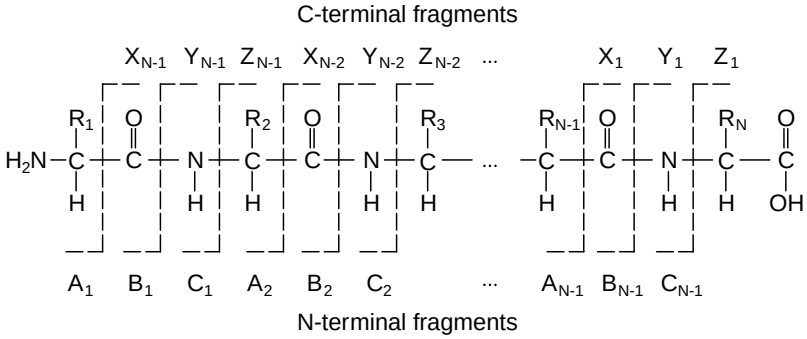


Fig 1-11. Fragmentation nomenclature (P. Roepstorff and J. Fohlman, Biomed. Mass Spec. 11 (1984) 601. K. Biemann, Biomed. Env. Mass Spec. 16 (1988) 99.)

The protein can be identified by comparing the masses of the precursor ion and PSD fragments with a computer-generated database.

2 Safety

The purpose of this chapter is to provide safety information and present all safety labels that are attached to the instrument.

Before using the Ettan MALDI-ToF instrument, also read Chapter 2 Safety instructions in the *Ettan MALDI-ToF Instrument Handbook*.

2.1 Safety labels

The following safety labels are attached to the Ettan MALDI-ToF instrument to warn the user of potentially hazardous conditions:

At the sample entry door

WARNING! PINCH HAZARD. Make sure that nobody is working at the sample entry lock when using the **Process:Close door** command in the **Control** module.

At the connector panel

CAUTION! Read the *Ettan MALDI-ToF Instrument Handbook* before connecting or disconnecting any cables, or opening the covers of the Ettan MALDI-ToF instrument.

2.2 Warnings, instrument handling

WARNING! Always exercise extreme caution when handling this instrument. The Ettan MALDI-ToF contains a power supply producing lethal high voltage of more than 20 kV DC inside the instrument.

WARNING! Do not use the Ettan MALDI-ToF in any other way than described in this User Manual. The protection provided by the instrument may be weakened if other operations are performed.

WARNING! HIGH VOLTAGE. Do not open instrument covers. Refer servicing to qualified service personnel.

WARNING! INVISIBLE LASER RADIATION. AVOID DIRECT EXPOSURE TO BEAM. CLASS 3B LASER PRODUCT. Do not open instrument covers. Refer servicing to qualified service personnel.

WARNING! HEAVY OBJECT. The Ettan MALDI-ToF weighs 152 kg. Contact Amersham Biosciences before moving the instrument.

3 System set-up

3.1 Required installations

The Ettan MALDI-ToF instrument and the PC should be installed and prepared by Amersham Biosciences personnel.

The Ettan MALDI Software should be installed after installation of Oracle software (Oracle Corporation) in the PC.

3.2 Start the PC and the instrument

1 Switch on mains power to Ettan MALDI-ToF. The instrument should be on for about 24 h before analysis, see the *Ettan MALDI-ToF Instrument Handbook*.

2 Switch on mains power to the PC.

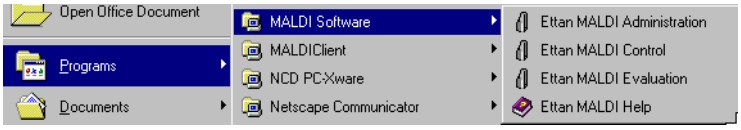
The Ettan MALDI Software consists of two modules: **Control** and **Evaluation**.

- The **Control** module is used for instrument control and for preparing an analysis.
- The **Evaluation** module is used for evaluation of results

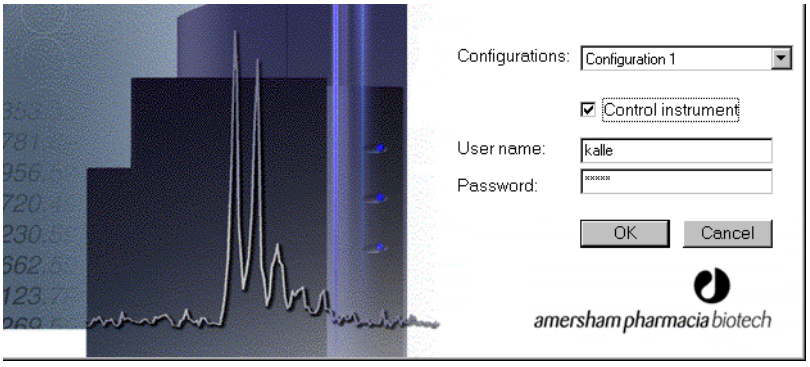
WARNING! HIGH VOLTAGE. When the software modules are started, the high voltage in the instrument will be turned on.

3.2.1 Start and log in to the Control module

To start the **Control** software, select the **Start** button in Windows NT and select **Programs:MALDI Software:Ettan MALDI Control.**



In the Login dialogue, enter user name and password. If the user wants to control the instrument, check the **Control instrument** box. Only one user can control the instrument during a session. Click **OK**.



3.2.2 Start the Evaluation module

To start the **Evaluation** software:

- select the **Start** button in Windows NT and select **Programs:MALDI Software:Ettan MALDI Evaluation.** This selection requires a login.
- or start the module from **Ettan MALDI Control** by selecting the command **Tools:MALDI Evaluation**. Login is not required.

3.2.3 Screen structure

The screen is divided into four main areas.

- **A:** Workspace. Shows the tree structure of selected samples, methods and results.
- **B:** Graph window. Shows an acquired MS spectrum, for example.
- **C:** Information window. Shows a mass list, for example.

Commands are selected from the menubar or toolbar (**D**). Tabs are available for selection of workspace and tree structure (**E**). Finally, favourite methods can be collected in the **Favourites** window (**F**).

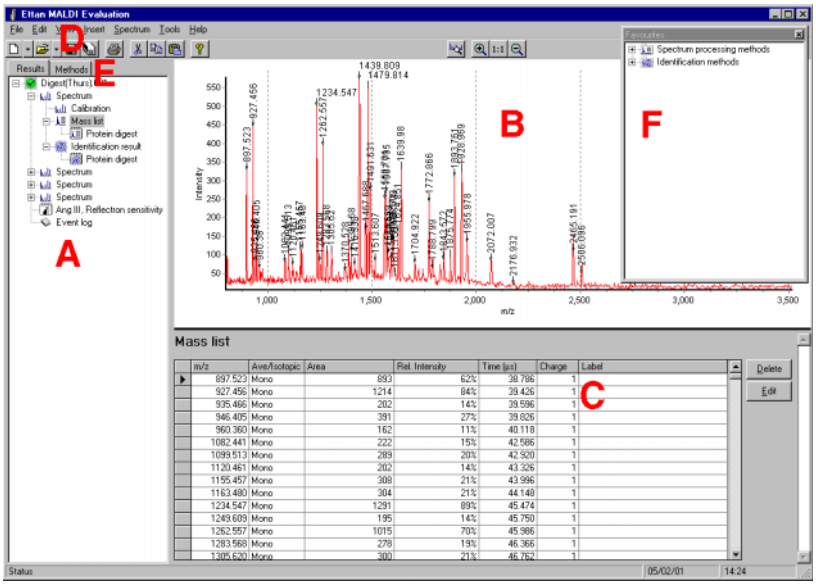


Fig 3-1. Software screen structure.

Many of the described commands in the Ettan MALDI-ToF User Manual can also be performed by using the right mouse button and select a command from the list.

3.2.4 Typographic conventions

Menu commands, the names of dialogue boxes, windows and option buttons are written in a **bold** typeface. Menu commands are written with the menu name and the command, separated by a colon.

For example: Select **File:Save as** to display the **Save as** dialogue. Locate the destination drive and folder and enter a file name.
Click on **Save**. This directs you to click on the **File** menu and select the

Save As command. The **Save As** dialogue called is displayed, in which you locate the destination folder for the saved file and give the file a name. You then click on the **Save** button called in the dialogue box to execute the save command.

3.3 Check instrument status

When the Ettan MALDI Control Software is running, check the instrument status and pressures in the **Status** bar in the lower end of the screen.

- In the **Status** bar, check that the **Pirani (mbar)** and **Magnetron (mbar)** pressure indications are decreasing. The operational Magnetron vacuum of below $1x10^{-6}$ mbar and Pirani pressure of below $1x10^{-2}$mbar should be reached within 24 hours.
- Check that the vacuum readings are stable before starting the experiment. When the vacuum readings are within the specification the **Instrument status** will state **Idle.**

- Check the lamps on the instrument cover:
  - When power is on, the **Power** lamp on the instrument cover lights.
  - When appropriate Magnetron vacuum is reached, the **Vacuum** lamp lights.
  - When the high voltage is on, the **Active** lamp lights.

3.4 System administration

A competent person at the laboratory should be appointed as System Administrator for the Ettan MALDI-ToF system. The System Administrator has access to removal of results, system configuration, user administration and to enzyme and modification editors. The System Administrator is also responsible for backup routines.

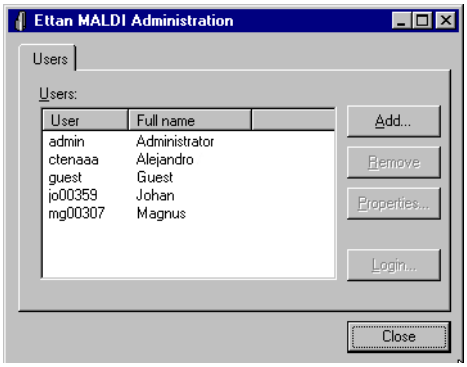
3.4.1 Add and remove users

1 Select the **Start** button in Windows NT and select **Programs:MALDI Software:Ettan MALDI Administration.**

2 Click the **Login** button and enter user name and password.

3 To add a user, click **Add.** To remove a user, select the user and click **Remove.**

4 Close the dialogue with **Close.**



3.4.2 User properties settings

To edit user properties, select **File:User Properties** from the menubar. Enter user data and click on **OK.**

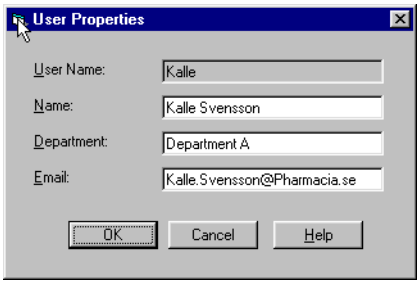


Fig 3-2. User properties entry box.

3.4.3 Change password

Select **File:Change password**. Enter old and new passwords and click on **OK**. This procedure can be performed by every user.

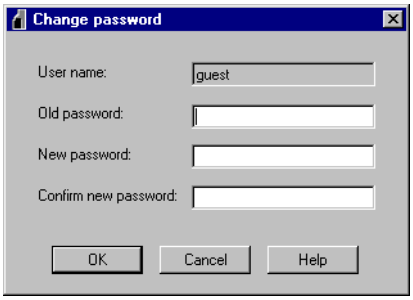


Fig 3-3. Password dialogue box.

3.4.4 Configure protein database

In the **Evaluation** module, select **Tools:Database editor**. A list of locally stored protein databases is shown. The nr database (NCBI non-redundant) is supplied and configured when the system is delivered. Databases can be added and deactivated by selecting them and clicking the **New** or **Delete** button. The databases must be in the FASTA format. After configuring the databases, click on **Close.**

The created list is used as an option in the Identification method.

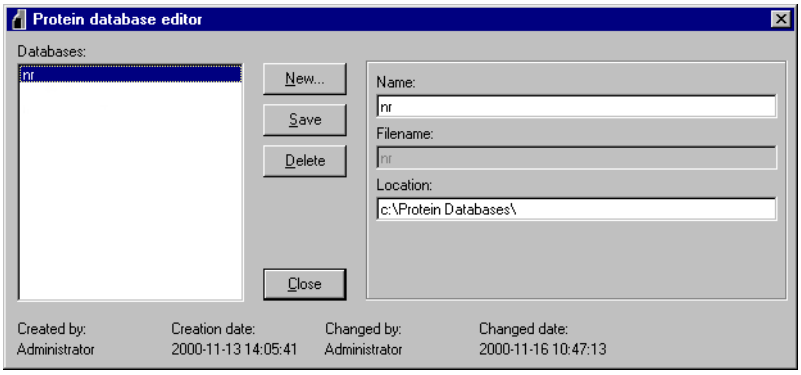


Fig 3-4. Protein database editor window

3.4.5 Configure proteolytic enzyme data

In the **Evaluation** module, select **Tools:Enzyme editor.** The data of the used proteolytic enzyme in peptide mass fingerprinting can be edited.

The created list is used as an option in the Identification method.

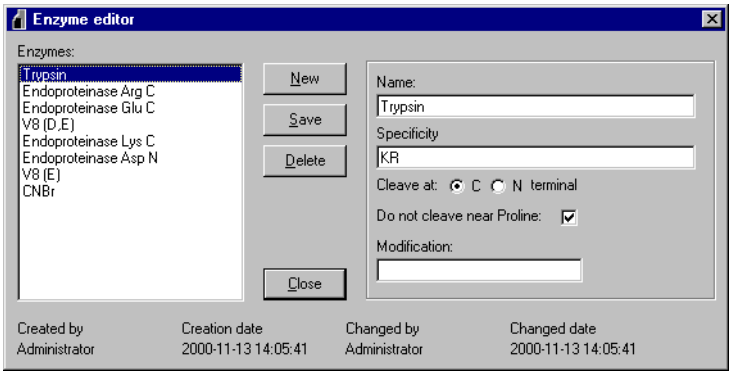


Fig 3-5. Enzyme editor window

When creating enzyme data, the enzyme **Specificity** has to be named with the standard amino acid one letter code:

Arginine:R, Alanine:A, Asparagine:N, Aspartic acid:D, Cysteine:C, Glutamic acid:E, Glutamine:Q, Glycine:G, Histidine:H, Isoleucine:I, Lysine:K, Leucine:L, Methionine:M, Phenylalanine:F, Proline:P, Serine:S, Threonine:T, Tryptophan:W, Tyrosine:Y, Valine:V.

Trypsin: Hydrolyses polypeptides on the carboxyl side of Arginine and Lysine. Enter **Specificity:** KR, and **Cleave at:** C terminal

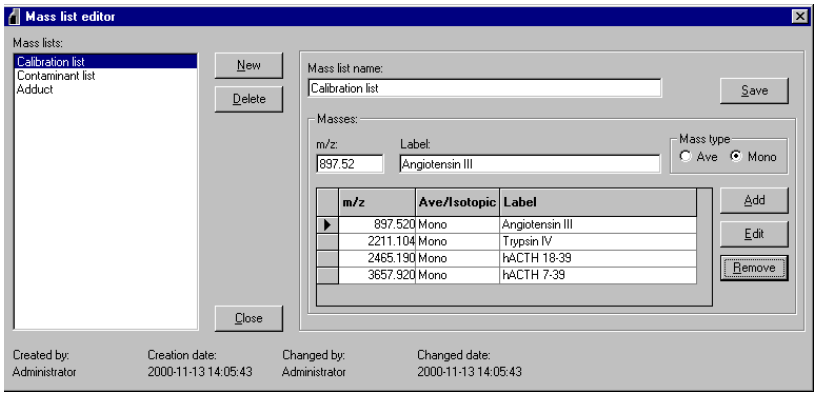
Chymotrypsin: Hydrolyses peptide bonds on the carboxyl side of aromatic and bulky non-polar side chains, Phenylalanine, Tyrosine, Tryptophan and Methionine. Enter **Specificity:** FYWM, and **Cleave at:** C terminal

Endoproteinase Asp-N: Cleaves at the C-terminal side of Aspartic acid or Cysteine. Cleavage at Glutamic acid has been reported. Enter **Specificity:**DC, and **Cleave at:** C terminal.

3.4.6 Create or edit a Calibration list

In the **Evaluation** module, select **Tools:Mass list editor.** In the **Mass list** window, select **Calibration list**. The list of used internal calibrants in peptide mass fingerprinting are created or edited in the **Masses** field by entering The **m/z** value, the expected **Mass type** (monoisotopic or average), and the **Label**. Only the four first found calibrants in the list will be used in the calibration of a spectrum. Save the list and close the dialogue.

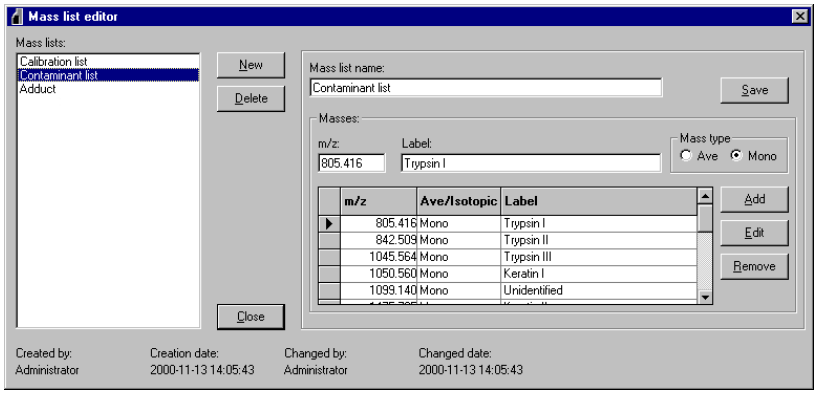
The created list is used as an option in the Spectrum processing method.



3.4.7 Create or edit a Contaminant list

In the **Evaluation** module, select **Tools:Mass list editor.** In the **Mass list** window, select **Contaminant list.** The list of known contaminants in the MALDI-TOF MS experiment is created or edited in the **Masses** window by entering the **m/z** value, the expected **Mass type** (monoisotopic or average), and the **Label**. Save the list and close the dialogue.

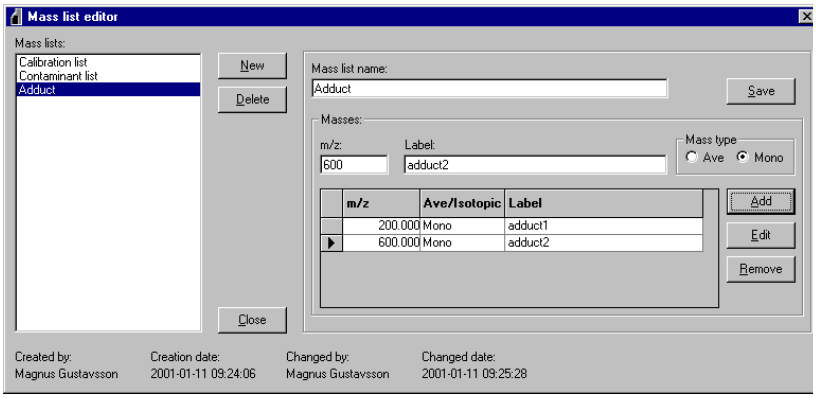
The created list is used as an option in the Spectrum processing method.



3.4.8 Create or edit a Adduct list

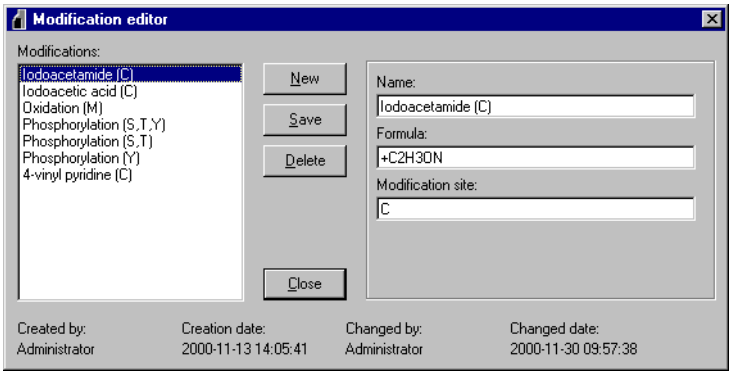
In the **Evaluation** module, select **Tools:Mass list editor.** In the **Mass list** window, select **Adduct list**. The list of known adducts in the MALDI-TOF MS experiment is created or edited in the **Masses** window by entering the **m/z** value, the expected **Mass type** (monoisotopic or average), and the **Label**. Save the list and close the dialogue.

The created list is used as an option in the Spectrum processing method.



3.4.9 Create or edit Modifications

In the **Evaluation** module, select **Tools:Modification editor.** A number of default modifications are available in the software. These are used as options in the Identification method.



Some of the default modifications are:

Modification	*Formula*	*Modification site*
Iodoacetamide (C)	+C2H3ON	C
Iodacetic acid (C)	+C2H2O2	C
Oxidation (M)	+O	M
Phosphorylation (S,T,Y)	+P03	STY
Phosphorylation (S,T)	+P03	ST
Phosphorylation (Y)	+P03	Y
4-vinyl pyridine (C)	+C7H7N	C

To create an new modification, enter in the **Formula** field an added chemical modification as e.g. "+C7H7" followed by the formula of a modification leading to a mass subtraction e.g. "-O". The mass of 7 carbons and 7 hydrogens will be added to, and the mass of one oxygen will be subtracted from, the specified amino acids in the protein database peptides before comparison with the measured peptides. The complete formula in this case is "+C7H7-O". The chemical formula of the first 86 elements in the periodic table can be used. Parenthesis are not allowed.

In the **Modification site** field, enter the one letter code (see Section 3.4.5) of the specified amino acids which are modified.

4 Sample preparation

4.1 Introduction

To achieve the accurate mass analysis needed for reliable investigations, the correct sample preparation is crucial. The protein or peptide should be transferred intact into the flight tube of the mass spectrometer. The presence of contaminants from previous techniques, the solutions used to dissolve the sample and the type of matrix used to contain the sample on the slide surface may all affect this process.

The MALDI-ToF technique is quite tolerant of certain contaminants, seeTable 4-1 . However, to achieve a high quality mass analysis, it is advisable to remove as many contaminants as possible or, at least, reduce them to acceptable levels.

Potential contaminant	*Levels not to be exceeded*
Phosphate buffers	< 20 mM
Tris buffer	< 50 mM
Detergents	< 0.1%
Alkali metal salts	< 1 M
Guanidine	< 1 M
Ammonium bicarbonate	< 30 mM
Glycerol	To be avoided
SDS	To be avoided
Sodium azide	To be avoided

Table 4-1. General tolerance levels for some common contaminants in MALDI-ToF analysis.

Reverse phase chromatography (RPC) is frequently used to remove salts and other low molecular weight substances. Peptides are bound to an RPC column containing 10-20 µm C18 medium. This process desalts and concentrates the sample, since the peptides bind in the presence of 0.1%TFA and are eluted in a small volume (1-4 µl) of 50% acetonitrile, 0.1%TFA. Columns are often handmade, by packing the RPC medium into microtips, or obtained as commercial products, such as ZipTip™.

4.2 Matrix

The surroundings of a protein or peptide should ensure that, when subjected to the intense light pulse of a laser, the molecule is transferred, in an ionized state, into the flight tube. To achieve this effect the sample is incorporated into crystals of a second material- the matrix. The sample-containing crystal absorbs the light pulse and the absorbed energy causes desorption of material from the crystal's surface, creating an ion cloud. If the desorbed material contains protein or peptide molecules, they will also enter the flight tube.

The following matrix compounds are commonly used:

- α-cyano-4-hydroxycinnaminic acid (4-CHCA)
  average and (exact mono) molecular mass =189.17 (189) Da
- Sinapinic acid (Sinapic acid: *trans*-3,5-dimethoxy-4-hydrocinnamic acid), average and (exact mono) molecular mass =224.22 (224) Da
- 2,5-dihydroxybenzoic acid (gentisic acid or DHB)
  average and (exact mono) molecular mass =154.12 (154) Da

Matrix	***Molecular mass***	***Ionization***	
		Peptides (< 4000 Da)	***Proteins***
4-CHCA	189 Da	+++	+
Sinapinic acid	224 Da	+	+++
DHB	154 Da	++	+

Table 4-2. Properties of some common matrices and their use.

If salts are present in the sample then satellite peaks, containing Na^+ or K^+ that replace protons as the charge donor, may be seen. Similar satellite peaks will also occur when using poor quality matrix substances.

4.3 Applying samples to the sample slide

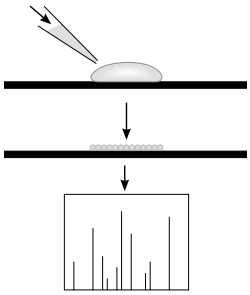
4.3.1 Dried droplet method

The dried droplet method is the first choice for most applications.

Note: *Do not use non-volatile solvents (e.g. glycerol, polyethylene glycol, DMSO, Triton X) with this method.*

1 Prepare a saturated solution of matrix in 50% acetonitrile in HPLC grade water with the addition of 0.1-0.5% TFA for 4-CHCA or 0.5-0.9% TFA for sinapinic acid. Homogeneity of the sample preparation can be approved by adjusting the TFA concentration within the indicated intervals.

2 Mix peptides or proteins with the matrix solution (ensure to avoid insoluble crystals) to a final concentration of > 20 nM for peptides and > 6 µM for proteins.



3 Apply 0.5 µl of solution to the sample slide.

4 Leave to dry at room temperature.

5 Insert the dried sample into the mass spectrometer and acquire the mass spectrum.

Following examples show crystals of sinapinic acid and 4-CHCA when the dried droplet method was used.

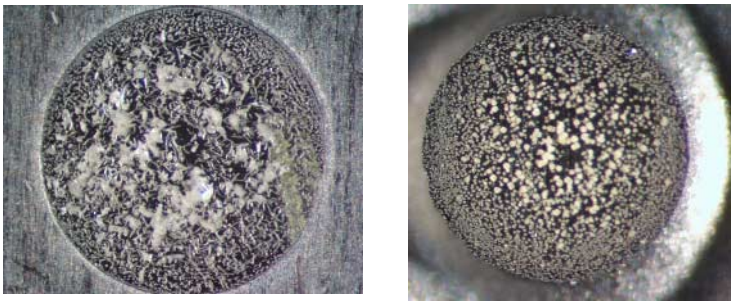
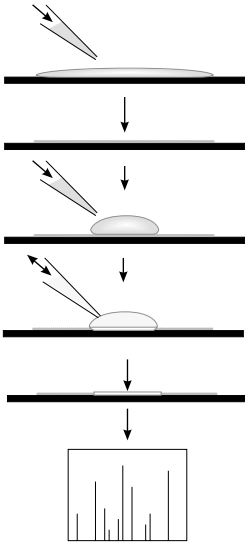


Fig 4-1. Crystals of sinapinic acid (left) and α-cyano-4-hydroxycinnaminic acid (4-CHCA) (right).



4.3.2 Modified dried droplet method

Suitable for use with 4-CHCA (peptides) and sinapinic acid (proteins).

1 Prepare a saturated solution of matrix in 50% acetonitrile in HPLC grade water with the addition of 0.1-0.5% TFA for 4-CHCA or 0.5-0.9% TFA for sinapinic acid.

2 Apply 0.5 µl of saturated matrix solution to the sample slide target.

3 Dry at room temperature to provide a layer of matrix.

4 Apply to the matrix layer 0.5 µl of peptide/protein in 50% ACN, TFA.

5 Dissolve the matrix crystals with the peptide/protein solution by mixing with a pipette.

6 Leave to dry at room temperature.

7 Insert the dried sample into the mass spectrometer and acquire the mass spectrum.

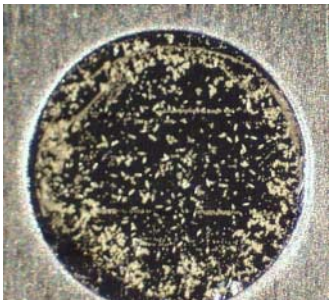


Fig 4-2. Crystals of 4-CHCA using modified dried droplet method.

4.4 Protocols

Further sample preparation protocols and applications are found in the *Ettan MALDI-ToF Method Handbook*.

For practical examples of sample preparations and mass spectrometry, see Chapter 10 .

4.5 Calibration

4.5.1 System calibration

System calibrations for the most common instrument modes are set during the installation of the Ettan MALDI-ToF system. It is, however, recommended to perform regular system calibrations for both the protein and peptide range. Peptide and protein calibrants are available from Amersham Biosciences.

Note: *Each combination of instrument mode (detection mode, accelerating potential) needs individual calibrations.*

The system calibrations are handled in the **Evaluation** module. Select the Calibration icon and use the right mouse button commands: **Save as:System calibration** or **Load system calibration**.

4.5.2 Within sample slide calibration (External calibration)

It may be convenient to have a calibrant mixture as a sample in one of the target positions of the sample slide. When analysing the molecular weight of a protein, it is recommended to have a protein calibrant mixture within the sample slide.

1 Calibrate the spectrum of the calibrant mixture.

2 Save the calibration as System calibration by using the right mouse button command **Save as:System calibration.**

3 Open the spectrum of the unknown sample.

4 Apply the System calibration to this spectrum using the right mouse button command **Load system calibration**.

4.5.3 Internal calibration

When the protocol of peptide mass fingerprinting is used, it is recommended to add at least two peptide calibrants to the sample before the mixing with the matrix. Internal calibration will increase the probability of obtaining correct results when searching external databases. An alternative to the addition of calibrants, is to use the peaks of the auto digestion of the proteolytic enzyme used. The creating of a Calibration list is described in Section 3.4.6 .

4.5.4 PSD spectrum calibration

When performing PSD, the mass of the precursor ion has to be accurately determined in the reflectron mode and the calibration saved as system calibration This mass is submitted as the ion gate mass in PSD mode. The fragments in PSD mode should now have a mass accuracy of approximately +/- 0.6 Da. PSD investigations are further described in Chapter 8 .

5 Preparing an analysis (Peptide mass fingerprinting)

5.1 Introduction

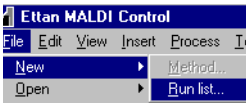
Three different types of method are needed for automatic acquisition, and evaluation:

- Acquisition method: controls the acquisition of a MS spectrum.
- Spectrum processing method: controls the filtration, calibration, and the peak detection of a MS spectrum.
- Identification method: sets the search parameters and performs the database search.

A Run list template (“Peptide mass fingerprinting”) is delivered with the Ettan MALDI Software containing a combination of methods (Method setup) with basic settings. Using this template is the fastest and easiest way to perform the first Ettan MALDI-ToF MS analysis.

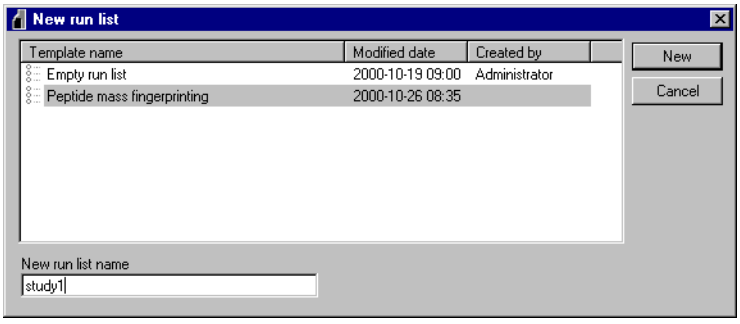
The creation of each individual method is described in detail later in this chapter. If the spectrum processing and database search will be performed manually only, the Acquisition method has to be created now.

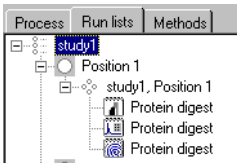
5.2 Using the run list template Peptide mass fingerprinting



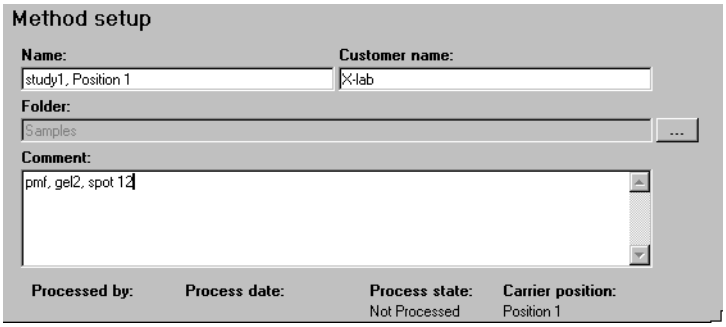
1 In the **Control** module, select the tab **Run lists** and then select **File:New:Run list.**

2 Select the template “Peptide mass fingerprinting”. Enter a new name in the **New run list name** box and click on **New.**

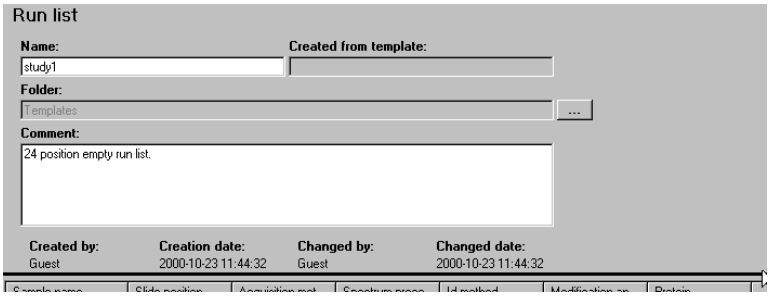




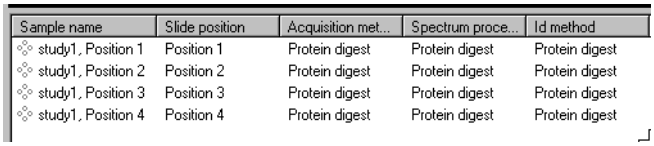
3 The run list will now be added to the workspace. Click on the plus-sign or double-click on the run list icon. A list of the sample slide positions is shown below. The sample identities (Method setup name) will be the name of the run list, followed by the position, e.g. study1, Position 1.



4 When the run list icon is activated, a dialogue with information for the run list is shown. It is here possible to decide where the run list is saved by using the button at the right side of the **Folder:** list.



5 Below the dialogue there is a list of sample identities and belonging methods.



6 Select **File:Save** to save the run list. Start the MALDI-TOF MS analysis as described in Chapter 6 .

5.3 Acquisition method templates

Three Acquisition method templates are available in the Ettan MALDI Software for Ettan MALDI-ToF.

- Full length protein
- Protein digest
- PSD

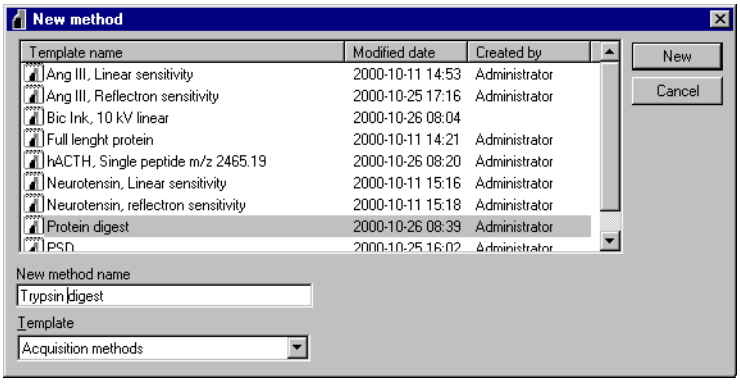
The selected Acquisition method template can be saved with a new name and modified according to the type of protein sample and the estimated molecular weights of the included proteins/peptides. For peptide mass fingerprinting the template **Protein digest** is recommended.

5.4 Creating an Acquisition method

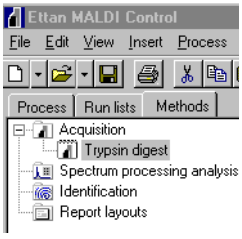
5.4.1 Creating an Acquisition method from a template

1 In the **Control** module select the tab **Methods** and then select **File:New:Method.**

2 Browse for, and select the desired method template. Enter a name for the new method in the **New method name** box.



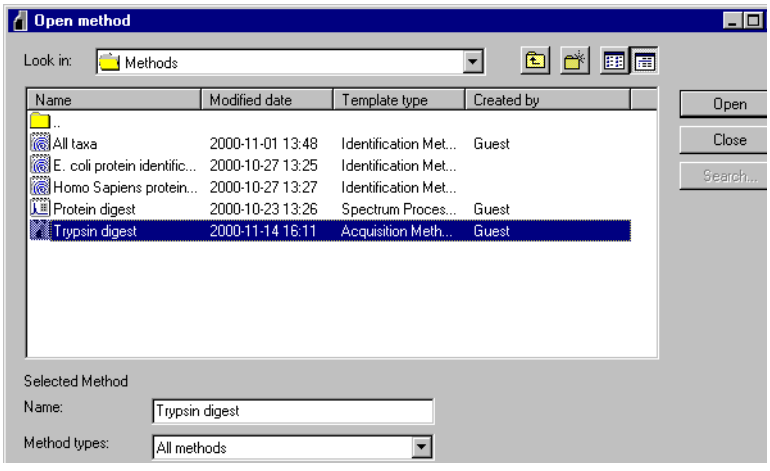
3 To add the selected method to the workspace, click on **New.**



5.4.2 Open an Acquisition method

1 Select the tab **Methods** and **File:Open:Method.**

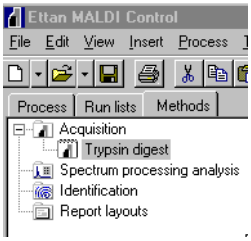
2 Browse the folders, select a method and click on **Open** to add it to the workspace.



3 If the method will be edited, select the method in the workspace and save it with a new name in with the **File:Save as** command. Browse for a folder and press **Save**.

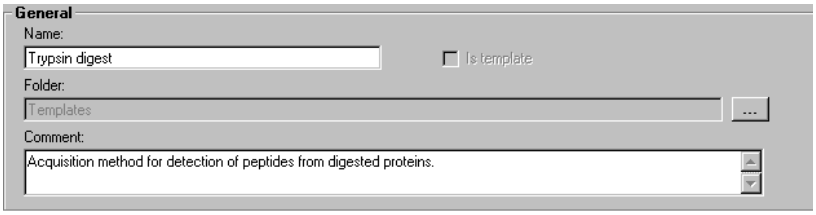
5.5 Editing an Acquisition method

1 Above the workspace, click the tab **Methods** and click on the Acquisition method which will be edited in the workspace.



2 The method parameters are organized in the dialogue as four different areas: **General, Instrument parameters, Control parameters** and **Acquisition parameters.**
A description of, and recommended start values when optimizing the method parameters, is included for each section. Select options and enter requested values and information in the boxes and fields.

5.5.1 Acquisition method: General

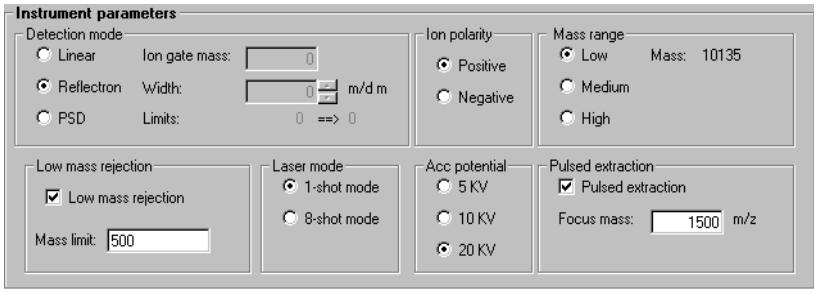


Method name: A new name can be entered.

Is template: If the method is a template, this box will be checked.

Folder: The folder where the method is saved can be changed with the button at the right.

5.5.2 Acquisition method: Instrument Parameters

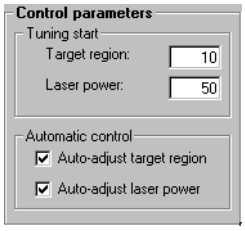


Category	*Parameter*	*Value/Option*
Detection mode	Linear	In linear detection mode, the reflectron is not in use and the detector is situated in the upper end of the flight tube. This mode is useful when analysing peptides/ proteins larger than 4000 Da due to improved sensitivity.
	Reflectron	Reflectron mode is recommended when studying peptides < 4000 Da due to the increased resolution.
	PSD	This mode is used when the objective is to study post-source decay. The mode includes the use of the reflectron and the timed ion gate.
	Ion gate mass	When PSD instrument mode is selected. Settings for the timed ion gate. The mass (*m/z*) value of the peptide peak to be studied.

Category	*Parameter*	*Value/Option*
	Width	The mass width for the preferred mass. The width should be $r \leq 100$ (where $r=M_1/(M_2-M_1)$). The limits are then shown.
Ion polarity	Positive or Negative.	Positive is default for peptides and proteins. Negative polarity is mainly used for nucleic acids.
Mass range	Mass range selection Low, Medium or High	The range should be set as low as possible. For peptides “Low” is default. A mass value is then shown in the **Mass** box.
Low mass rejection	On (ticked box)/off	Low mass rejection **on** reduces the flow of smaller compounds which may otherwise saturate the detector.
	Mass limit	Recommended value is 500 to avoid peaks from matrix compounds.
Laser mode	1 or 8-shot mode	8-shot mode means 8 shots/sec and leads to a reduced time to accumulate a specified intensity. 1-shot mode is however recommended as a start value.
Accelerating potential	5, 10 or 20 kV	20 kV is recommended for peptide mixtures. However, a lower value may increase resolution.

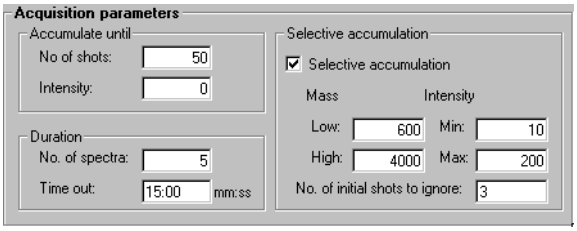
Category	*Parameter*	*Value/Option*
Pulsed extraction	On (ticked box)/off	Refers to the function of pulsed extraction from the ion source. The default is to have pulsed extraction **on**.
	Focus mass	Is set according to the estimated molecular weight. The maximum value is 10 000 Da. For peptides, a value between 1500-2500 is recommended.

5.5.3 Acquisition method: Control Parameters



Category	*Parameter*	*Value/Option*
Automatic control Auto-adjusts target region and laser power	On (ticked box)/off.	"On" means auto tuning. The response specifications are set in the dialogue **Selective accumulation.**
Tuning start	**Target region**	Recommended start value is 10-30.
	Laser power	Start at 20 is recommended.

5.5.4 Acquisition method: Acquisition parameters



Category	*Parameter*	*Value/Option*
Accumulate Until.	Parameters for spectrum acquisition	
	No. of Shots Number of shots to accumulate.	50-100 shots per spectrum is recommended for protein digests.
	Intensity The level of intensity to achieve.	As an alternative to number of shots, a level of accumulated intensity for a spectrum can be used.
Duration		
	No. of spectra Number of spectra to acquire.	Only the first spectrum will be automatically evaluated. However, two spectra for each sample, can be a recommendation.
	Time Out Duration time in minutes.	A maximum time of 10 minutes is recommended to avoid delays for coming samples.

Category	*Parameter*	*Value/Option*
Selective Accumulation	On (ticked box)/off	If Selective accumulation is **on,** the auto tuning function works according to a principle of varying the laser power and the position of the target to find a response within the m/z intensity window. The tuning optimization is based on the highest peak in the specified tuning window.
	Mass - Low	A recommended value, given by the templates, is 600 for peptide mode.
	Mass - High	Default value in the template is 4000 for peptide mode.
	Intensity - Min.	5-10 is recommended for peptides and 2-5 for full-length proteins.
	Intensity - Max.	100-200 is recommended.
	No of initial shots to ignore	3-5 is recommended.

5.5.5 Save an Acquisition method

Save the method using **File:Save**. To save the method as a template, select **File:Save as template.**To add the method to the **Favourite** folder, select **Tools:Add to favourites**.

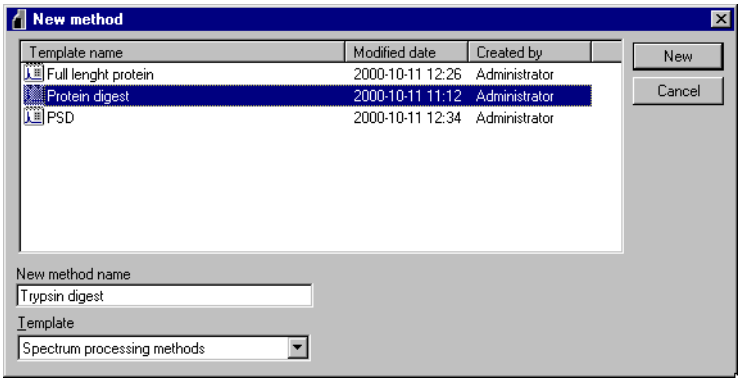
5.6 Creating or editing a Spectrum processing method

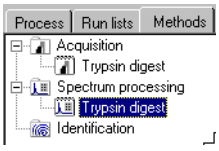
Three Spectrum processing method templates are available in Ettan MALDI Software:

- Full length protein
- Protein digest
- PSD

1 In the **Control** module, select the **Methods** tab and **File:New:method**.

2 Select a template, enter a new name and click on **New**. For peptide mass fingerprinting the method template **Protein digest** is recommended. The method is now added to the workspace.





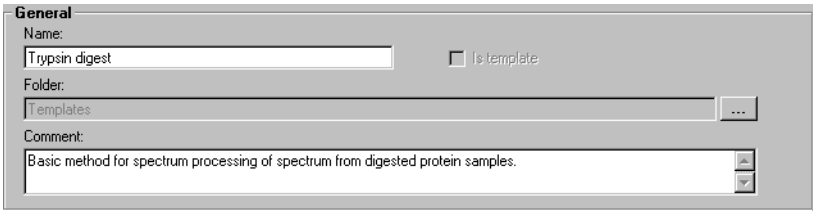
3 Click on the Spectrum processing method icon.
The Spectrum processing method will contain information regarding:

- Parameters for the filtration of a spectrum
- Parameters for the internal calibration of a spectrum
- Parameters for the peak detection of a spectrum
- Parameters for the mass list adjustments

The method, created from the template, can be further optimized for a specific experiment by varying parameters.

To edit a previously created method, select **File:Open:Method**. Select a method and click **Open**. Save the method with a new name in preferred folder with the **File:Save as** command, before editing.

5.6.1 Spectrum processing method: General



Is template: If the method is a template, the box is checked.

Folder: The folder of storage can be selected by using the button at the right.

Comments: Additional comments can be entered.

5.6.2 Spectrum processing method: Filter

Filters
Filters are commonly applied to time-of-flight mass spectra to improve the appearance of a spectrum and to extract peaks from poorly resolved signals.

Note: All filters distort the mass spectrum and should therefore be used with care. Using the wrong parameters can lead to increased error in the extracted masses. It is recommended that the filters are used with caution and preferably only for display purpose.

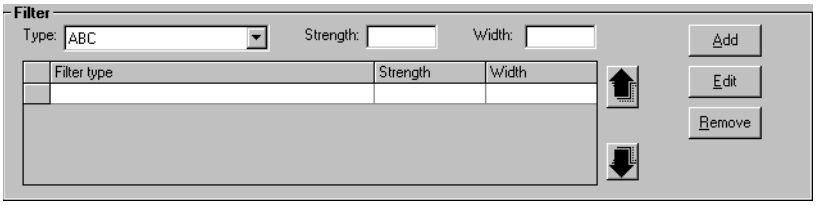
See Chapter 11 , for a more detailed description of the terms Filters, Smoothing filters, High-pass filters, and ABC correction.

Smoothing filters
Smoothing filters are usually applied to noisy data to reduce the high-frequency noise associated with the signal, i.e. they are a type of low-pass filters. By applying few points wide smoothing filter the quality of peaks can be improved. However, if the width is increased the resolution deteriorates.

High-pass filters
High-pass filters are usually applied to low resolution data to enhance the high-frequency signal and remove the low frequency background. By applying a high-pass filter in combination with a smoothing filter the quality of peaks can be improved.

Adaptive Background Correction (ABC)
ABC filters are usually also applied to low resolution data to enhance the high-frequency signal and remove the low frequency background. By applying an ABC filter in combination with a smoothing filter the quality of peaks can be improved.

Values



Category	*Value/Option*
Filter	
Type	ABC High-pass Smoothing
Strength	1-10, not valid for smoothing filters.
Width	1-100
Order	Select the order of filtration with the arrow buttons.

Select **Type** of filter, enter **Strength, Width** and click **Add.**

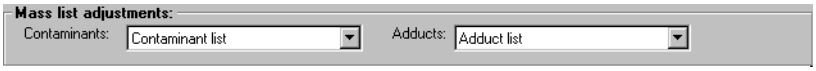
5.6.3 Spectrum processing method: Internal calibration

Category	*Value/Option*
Calibrants	Select Calibration list in list box. The list is created in the **Mass list editor.** See Section 3.4.6
Error (Da)	Enter a value in Dalton. A value between 3-5 is recommended.
Min. signal to noise ratio	A start value of 4 is recommended.

5.6.4 Spectrum processing method: Peak detection

Category	*Value/Option*
Mass range	Enter ranges. For peptide mass fingerprinting 600-4 000 is an appropriate start value.
Mass error A_o	A value used in protein database search. 0.3 Da is appropriate.
Mass error Ave	A value used in protein database search. 1 Da is appropriate
List box	Select unit of the error values.
Resolution	6000 is recommended to avoid detecting background peaks.
Min. signal to noise ratio	3 is an appropriate start value.

5.6.5 Spectrum processing method: Mass list adjustments



Category	*Value/Option*
Contaminants	Select in the list box a Contaminant list. The list is created and edited in the **Mass list editor.** See Section 3.4.7 .
Adducts	Select in the list box an Adduct list.The list is created and edited in the **Mass list editor.** See Section 3.4.8 .

Select lists of known contaminants and adducts in the **Mass List Adjustments** section to exclude these peaks from the created peak list.

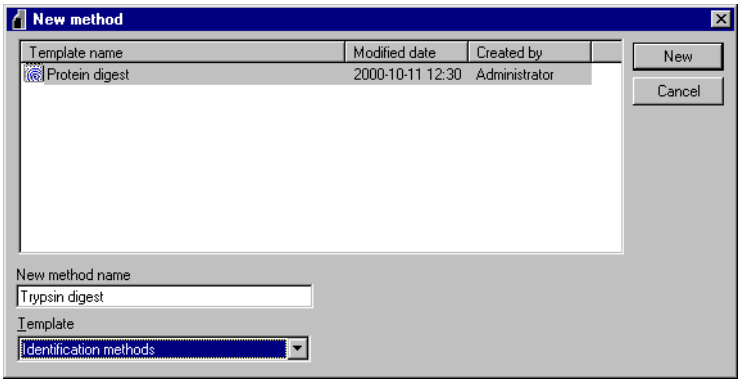
5.6.6 Save a Spectrum processing method

To save the method with the current name and folder, select **File:Save.** To save the method in an alternative folder, and/or with a new name, select, **File:Save as.** To save the method as a template, select **File:Save as template.** Add the method to the **Favourites** window with the command **Tools:Add to favourites.**

5.7 Creating an Identification method

There is a method template named **Protein digest** available that can be used in peptide mass fingerprinting.

In the **Control** module, click the **Methods** tab and select **File:New:Method**. Select a template(e.g. Protein digest), enter a new name and click on **New**. The Identification method is added to the workspace and is opened with a click on the icon.

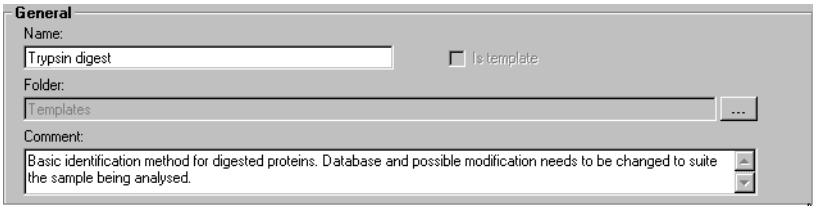


To edit a previously created method, select **File:Open:Method**. Select a method and click **Open**. Save the method with a new name in preferred folder with the **File:Save as** command, before editing.

The Identification method will contain information regarding:

- Requested database
- Parameter settings for search criteria.
- The type of, and occurrence of modifications

5.7.1 Identification method: General

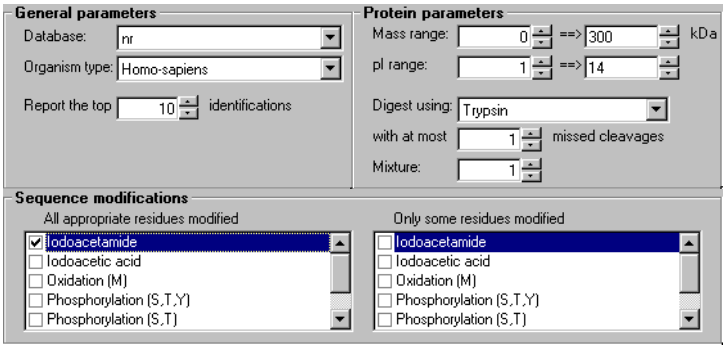


Is template: If the method is a template, the box will be checked.

Folder: The folder of storage can be selected by using the button at the right.

Comments: Additional comments can be entered.

5.7.2 Identification method: Parameters



Category	*Value/Option*
Database	Specify the sequence database to be searched e.g. NCBI's nr. Available databases, if they are in the FASTA format, can be connected in the **Protein database editor** by selecting **Tools:Database editor** (see Section 3.4.4)
Organism type	Is also named the taxonomic category and is a phylogenic tree with which the user can specify the origin of the sample protein. If the origin is unknown, “All Kingdoms” or “All taxa” can be selected.
Report the top.	Enter the requested number of top proteins to be reported in the list box.

Category	*Value/Option*
Mass range	The mass range for the analysed protein can be specified. It is, however, recommended to use a wide range since the analysed protein may have been fragmented or be a subunit of a larger protein.
pI range	The range of isoelectric point (pI) can be entered. A wide range is, however, recommended.
Digest using	Select the used proteolytic enzyme from the list. The enzyme list is created in the **Enzyme editor.** See Section 3.4.5 .
with at most.....missed cleavages	The number of estimated missed cleavages can be entered in this section. A value >2 is not recommended.
Mixture	If it is known or suspected that the sample contains more than one protein, a value can be selected in this section.
Sequence modifications	Specify modifications by ticking the boxes. The modifications can be set for all the residues or only for some residues. The modifications are edited and created in the **Modification editor**. See Section 3.4.9 .

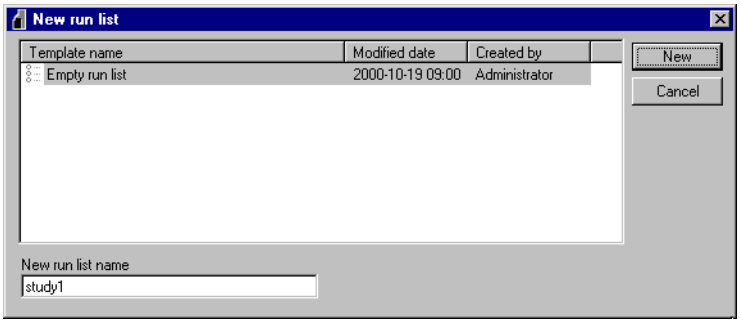
5.8 Creating run lists

A run list contains the information for each sample on a sample slide and the methods which will be applied to it. The defined process for each sample is called a Method setup.

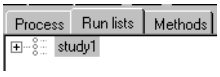
5.8.1 New run list

1 To create a new run list, select the tab **Run lists** in the **Control** module and **File:New:Run list.**

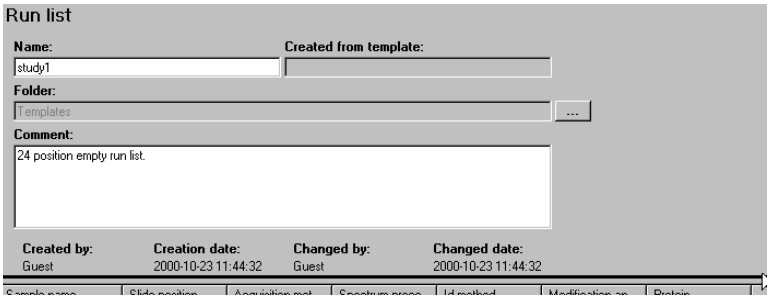
2 Select a template, enter a new name in the **New run list name** box, and click **New**.



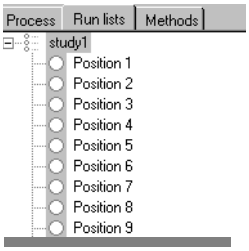
3 Click on the tab **Run lists** above the workspace and the selected run list will be shown in the workspace.
Click on the run list icon.



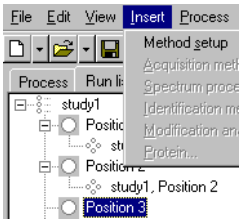
4 A **Run list** dialogue is shown in the graph window. The folder of storage can be changed with the button at the right of the **Folder** list. It is also possible to enter some comments of the run list in the **Comment** field.



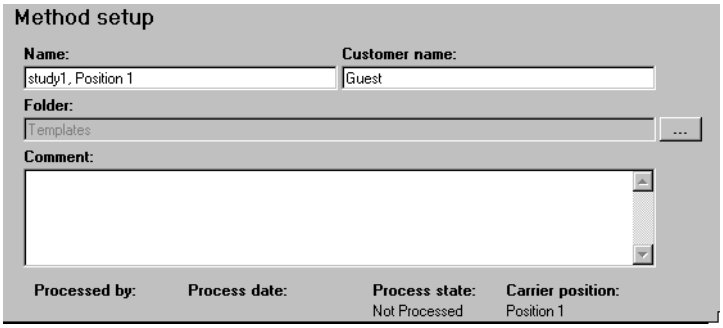
5 Double-click on the run list icon or click on the “plus” sign. A list of numbered positions will be shown.



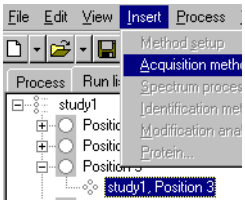
Inserting Method setups and methods



1 Before adding methods to a position it is necessary to add a Method setup to the position. To insert a Method setup for a position, select the Position icon, and select **Insert:Method setup.** The identity of the Method setup will be the run list name followed by the position (e.g. study1, Position 2).

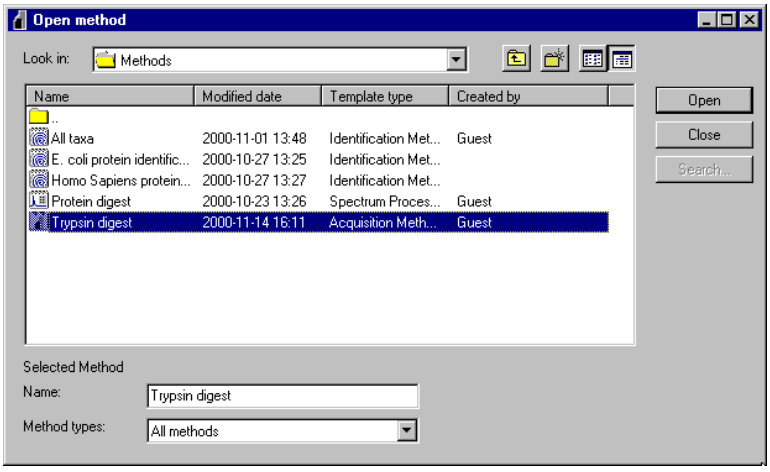


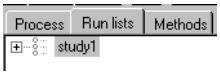
Methods can be inserted to one Method setup or to all added Method setups within a runlist.



2 To insert an Acquisition method to one Method setup, select the Method setup icon, and select **Insert:Acquisition method.**

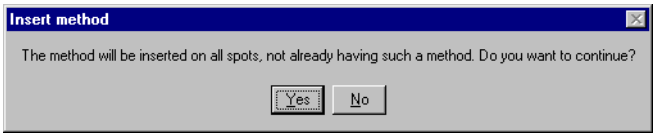
3 In the **Open method** dialogue, browse for the folder, select a method and click **Open.**

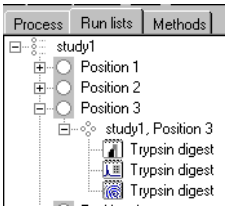




4 To insert an Acquisition method to all Method setups, select the run list icon in the workspace and select **Insert:Acquisition method.**

5 Click **Yes** to accept the command.





6 Insertion of Spectrum processing and Identification methods are performed in the same way as for Acquisition methods. All inserted methods will be shown in the tree structure.

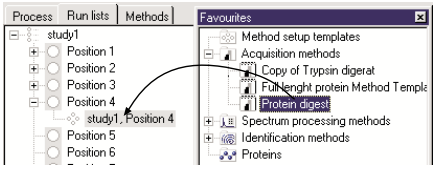
Note: *To replace an incorrectly inserted method, the incorrect method must first be excluded from the Method setup. Select the method, click the right mouse button and select* **Delete**.

7 When all positions are entered, save the run list with **File:Save** or **File:Save as**. The run list can also be saved as a template with the **File:Save as template** command.

Drag-and-drop inserting of methods

1 An alternative to the insert function is to use drag-and-drop from the **Favourites** window.

2 Select the desired **Acquisition Method** in the **Favourites** window. Drag and drop it on the Method setup icon or on the Run list icon. A "plus" sign will be shown when the action is performed correctly.



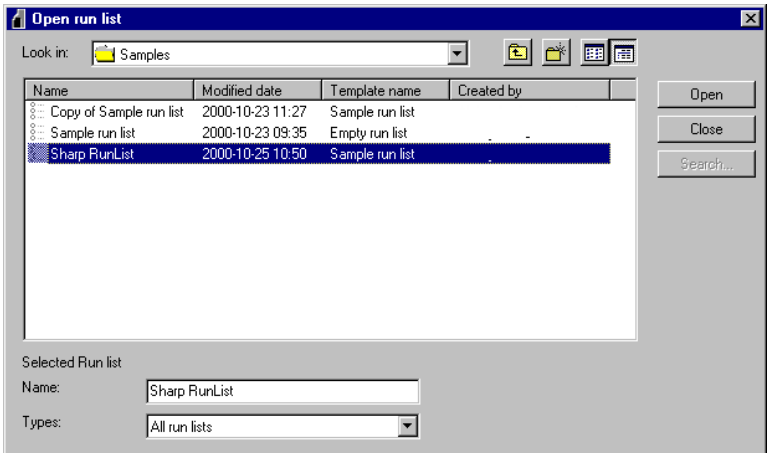
3 The same procedure can then be performed for the other methods.

4 When all positions are entered, save the run list with **File:Save** or **File:Save as**

5.8.2 Open Run lists

This section describes how to open a previously used run list. The run list can then be edited and saved under a new name.

1 Select in the **Control** module the tab **Run lists** and **File:Open:Run list.** Select location in the **Look in** list box. Select a run list and click on **Open.**



2 Edit the run list in the workspace and save it under a new name with the **File:Save as** command.

6 Analysing samples

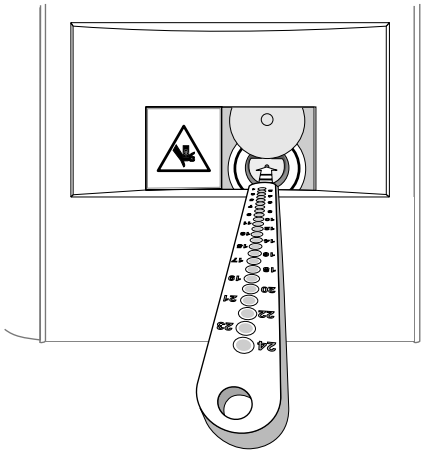
6.1 Loading a sample slide

1 In the **Control** module, choose **Process:Open door** to open the sample door.
The **Status** bar in the lower end of screen will indicate **Idle** and **Door is opened**. The slide tray is moved from the target position to the sample entry lock, which is then vented to atmosphere. This takes about 30 seconds.

2 When the door to the sample entry lock has opened, remove any sample slide already in the instrument.

3 Insert the sample slide into the sample entry lock. Press the slide firmly but gently until it stops.The samples must be dry before introducing into the instrument, otherwise the pump-down time will be extended.

Note: *Use lint and powder-free gloves or a pair of tweezers to avoid contaminations.*



Note: *To minimize moisture in the flight tube, do not leave the door open for long periods of time. Close the door even if a sample slide is not inserted.*

WARNING! Make sure that nobody is working at the sample entry lock when choosing the **Process:Close door** command.

4 Choose **Process:Close door** to close the sample door. The following will happen (the closing procedure takes 1-2 minutes):

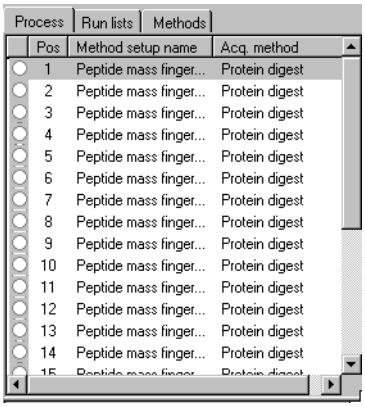
- The door closes.
- The sample entry lock is evacuated by the vacuum system. If operational vacuum in the sample entry chamber is not reached within 2 minutes, an error message is displayed and the door opens.
- The sample slide is moved into the target position.
- The **Vacuum** indicator is lit.

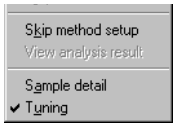
6.2 Opening and submitting a run list

1 In the **Control** module, select the tab **Run lists** and **Process:Open and submit run list**.

2 Browse for the requested run list, select the run list and click on **Open**.

3 The run list is now added to the **Process** workspace.


Process
Run lists
Methods
Pos
Method setup name
Acq. method
1 Peptide mass finger... Protein digest
2 Peptide mass finger... Protein digest
3 Peptide mass finger... Protein digest
4 Peptide mass finger... Protein digest
5 Peptide mass finger... Protein digest
6 Peptide mass finger... Protein digest
7 Peptide mass finger... Protein digest
8 Peptide mass finger... Protein digest
9 Peptide mass finger... Protein digest
10 Peptide mass finger... Protein digest
11 Peptide mass finger... Protein digest
12 Peptide mass finger... Protein digest
13 Peptide mass finger... Protein digest
14 Peptide mass finger... Protein digest



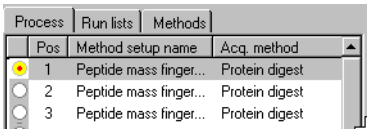
4 To exclude a sample position from the run, select the position's row, and use the right mouse button command **Skip method setup**.

6.3 Start a process

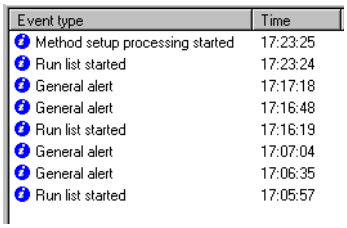
Check that Instrument status states **Idle**. Start the process by selecting **Process:Start** or use the Start arrow in the Toolbar. When analysis has started the Status will be **Running**.

6.4 Monitoring the process and the results

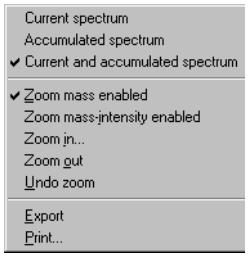
1 The sample in process is marked with yellow and red. An approximation of remaining time for the current sample slide is shown below the process list.

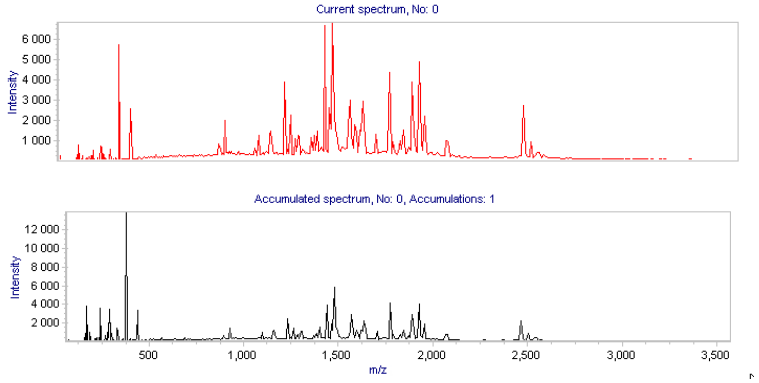


2 A list of events is shown below the process list window. The list notifies the time and type of the event.



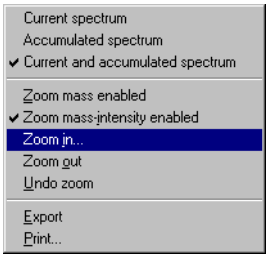
3 The current and the accumulated MS spectrum for the sample in process is shown in the graph window. To select only current or accumulated spectrum, use the right mouse button.

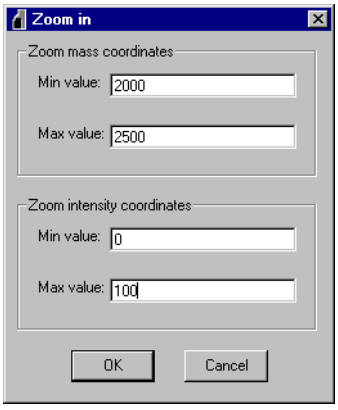




4 To zoom the spectrum, place the cursor at the beginning of the requested interval, hold down the left mouse button and drag a line to the end of the interval. A zoomed spectrum will be shown when the mouse button is released.

5 An alternative to zooming is to use the right mouse button, select **Zoom in** and enter the interval in mass and intensity directions.

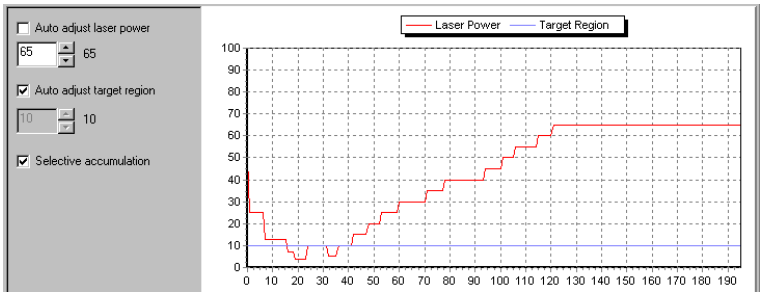




6 Zooming out is done in the same way by selecting the **Zoom out** command.

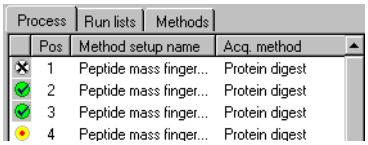
7 To view information for the current sample, select **View:Sample detail.** The Method setup is shown in the information window.

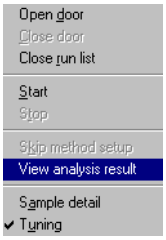
8 To view tuning status, select **View:Tuning.**



Tuning conditions (manual/automatic) are set in the Acquisition method editor. See Section 5.5.3 . The tuning can, however, be monitored and it is also possible to change to manual tuning by unchecking the boxes, and optimize for the best intensity. Values are entered in the white boxes and the measured values will be at the right side of the boxes. When automatic tuning is used, the software searches for an intensity within specified levels.

9 A sample which has been processed with all the inserted methods is marked with a ticked green circle.





10 To view results of a sample, select the sample, use the right mouse button and select **View analysis result.**

11 When all samples on the sample slide have been processed, select **Process:Open door.** Take out the sample slide and close the door.

12 To find all results, start the **Evaluation** module and select **File:Open results.**

7 Evaluating mass spectra

7.1 Protein identification after automatic evaluation

1 Perform the steps in Section 7.2.1.

2 Use the “plus” sign or double-click until the icon for identification is reached.

3 Perform the steps in section 7.2.3.

7.2 Viewing automatic evaluated MS results

If analysis methods were inserted in the run list before process, all evaluations have now been performed.

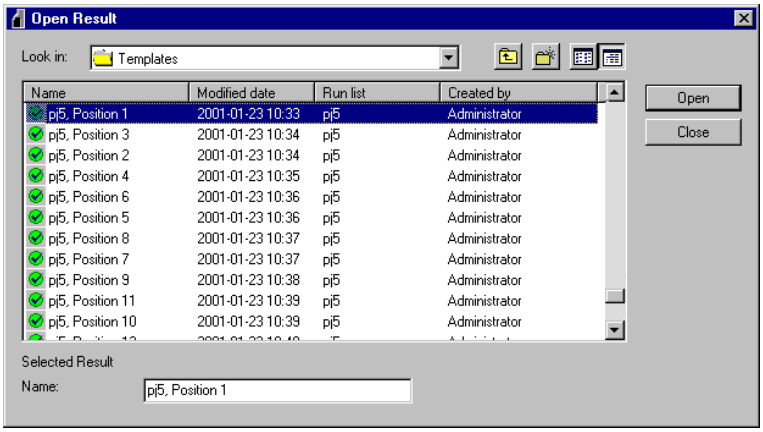
7.2.1 Open result

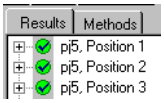
1 To view results for a sample, select the program module **Evaluation.**

2 Click on the **Results** tab and select **File:Open:Result.**

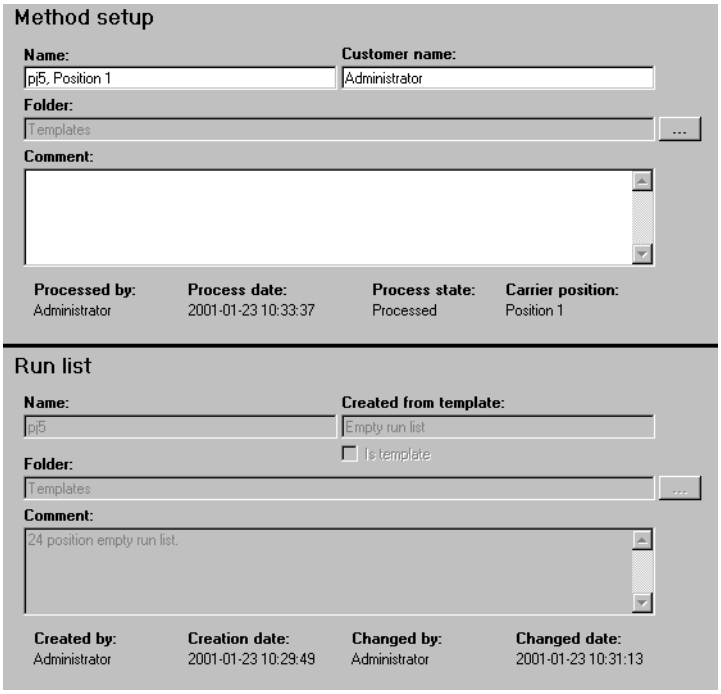
3 Browse for a requested sample. Select the sample and click on **Open.**

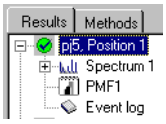
4 To open several samples to the workspace, repeat the procedure.


Open Result
Look in:
Templates
Name
Modified date
Run list
Created by
pj5, Position 1 2001-01-23 10:33 pj5 Administrator
pj5, Position 3 2001-01-23 10:34 pj5 Administrator
pj5, Position 2 2001-01-23 10:34 pj5 Administrator
pj5, Position 4 2001-01-23 10:35 pj5 Administrator
pj5, Position 6 2001-01-23 10:36 pj5 Administrator
pj5, Position 5 2001-01-23 10:36 pj5 Administrator
pj5, Position 8 2001-01-23 10:37 pj5 Administrator
pj5, Position 7 2001-01-23 10:37 pj5 Administrator
pj5, Position 9 2001-01-23 10:38 pj5 Administrator
pj5, Position 11 2001-01-23 10:39 pj5 Administrator
pj5, Position 10 2001-01-23 10:39 pj5 Administrator
Open
Close
Selected Result
Name:
pj5, Position 1

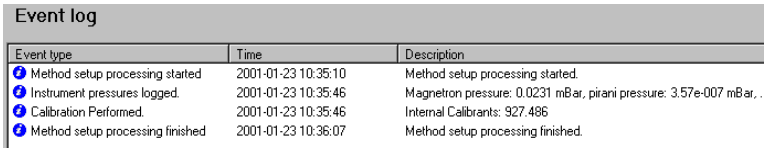


5 The selected samples list will be shown in the workspace. Click on the sample icon and information of the method setup and run list is shown.



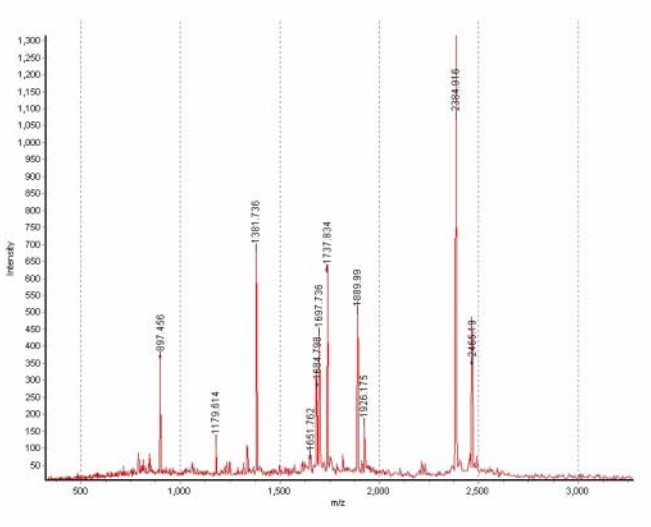


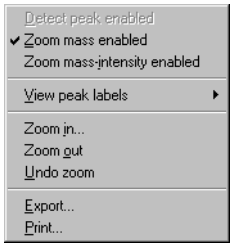
6 Double-click on the requested sample or use the “plus” sign The tree structure shows that A spectrum has been acquired using the Acquisition method PMF1. An event log is stored in the same structure. To view the event log, click on the event log icon and it will be shown in the information window.



7.2.2 View and edit spectra and mass lists

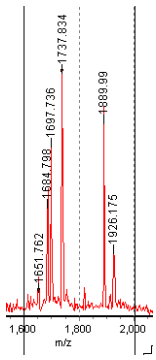
1 To view a spectrum, click on the spectrum icon.

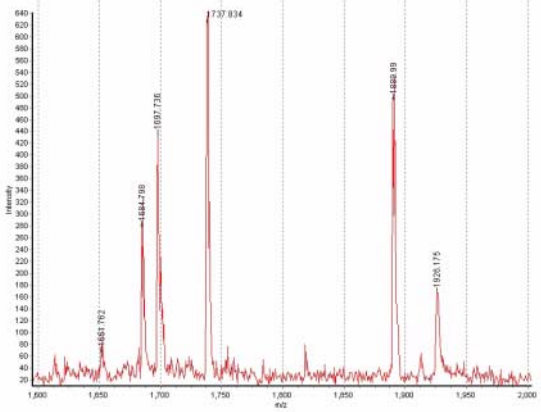




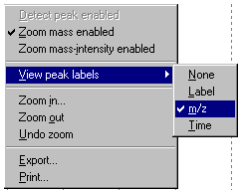
2 To zoom a specific part of the spectrum, select the right mouse button command **Zoom mass enabled**.

3 Place the cursor at the beginning of the interval, hold down the left mouse button and drag a line to the interval end. The procedure can be repeated to obtain further zooming. To zoom out, use the right mouse button and select **Zoom out** or **Undo zoom**.

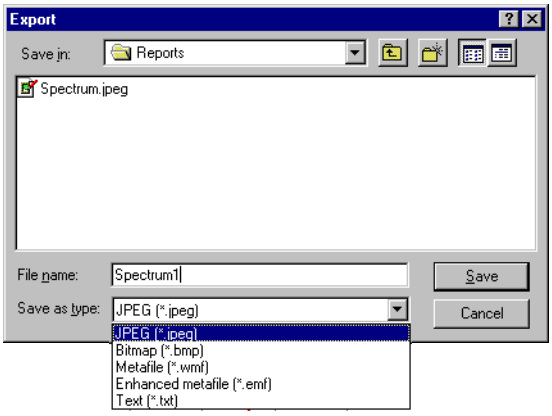




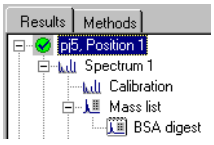
4 The Intensity range is automatically set to the highest peak in the m/z range. To zoom a spectrum in the Intensity direction, select the right mouse button command **Zoom mass-intensity enabled**. Drag a line with the mouse cursor to a preferred intensity interval.



5 To view different peak labels, select the right mouse command **View peak labels** and select labels **None**, **Label**, **m/z** or **Time** (µs). Spectra can be printed using the **Print** command and exported using the **Export** command. The available export picture formats are: JPEG, Bitmap, Metafile and Enhanced metafile.

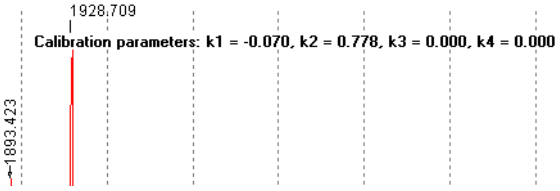


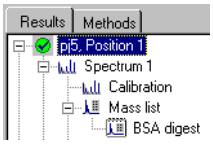
6 To export the spectrum in a text format, select **Save as type:Text**.This creates a two column ASCI file with m/z and intensity. Only the currently displayed m/z range is exported.



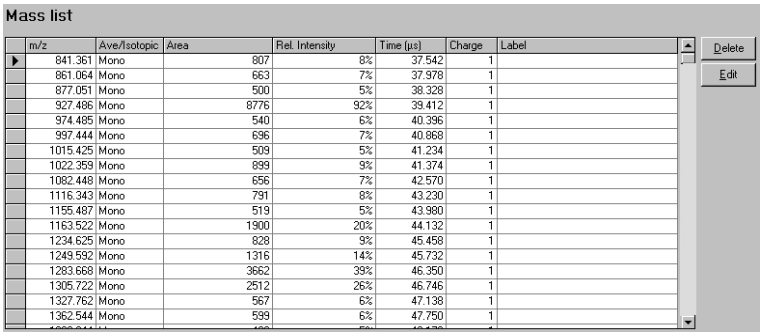
7 To view the created results from the current spectrum, double-click on the spectrum icon. The tree structure shows that the spectrum has been calibrated, and a Mass list has been produced using the Spectrum processing method “BSA digest”.

8 To view the calibration results, click on the **Calibration** icon. Detected calibrants in the spectrum are labelled and the calibration parameters are displayed. To add calibrants, see Section 7.3.2.



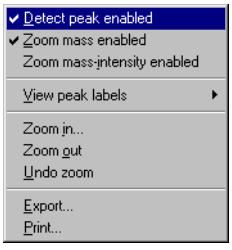


9 Click on the **Mass list** icon and the generated Mass list will be shown in the information window.



The list includes detected peak masses (m/z), whether the peaks were detected as monoisotopic or average masses. The peaks are presented as areas and relative intensities (% of the highest peak). The **Time** column is the flight time in µs. The assumed charges are usually +1 in MALDI-TOF experiments.

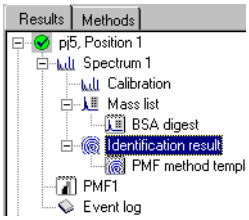
10 To exclude masses, select a row and use the **Delete** button. Peaks can be labelled by entering a text in the **Label** column and click on **Edit**. To sort the list, click one of the column heads.



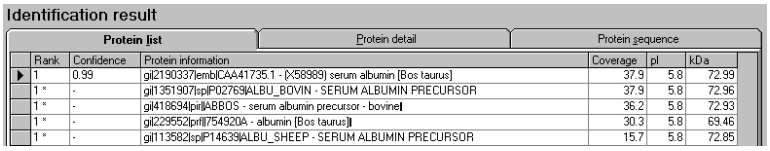
11 Not detected peaks can be added to the list by activating the right mouse button command **Detect peak enabled**. Click with the mouse cursor on a peak in the spectrum. This peak will be added to the Mass list. When all requested peaks are detected, deselect the command.

12 To print the Mass list, select **File:Print** or **File:Print preview** followed by **Print**.

7.2.3 View Identification results

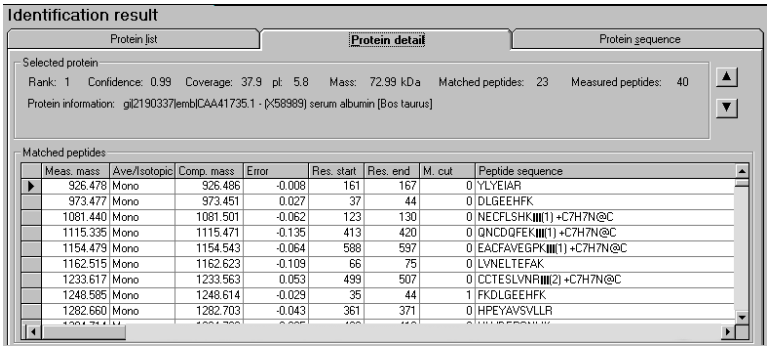


1 To view a result summary of protein identification, click on the Identification result icon. The used Identification method in this experiment was named “PMF method template”. To view a a summarized table of the top protein candidates, select the tab **Protein list**. To print the identification results, select **File:Print**.

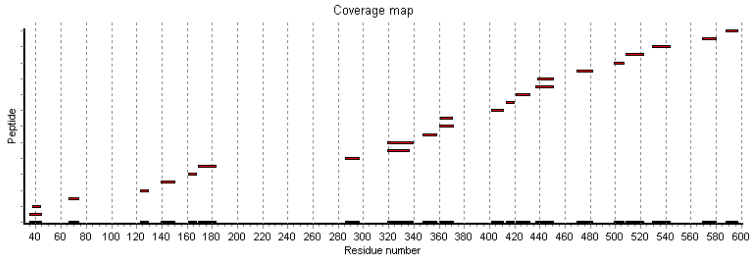

Identification result
Protein list
Protein detail
Protein sequence
Rank Confidence Protein information Coverage pI kDa
1 0.99 gi|2190337|emb|CAA41735.1 - (X58989) serum albumin [Bos taurus] 37.9 5.8 72.99
1 * - gi|1351907|sp|P02769|ALBU_BOVIN - SERUM ALBUMIN PRECURSOR 37.9 5.8 72.96
1 * - gi|418694|pir||ABBOS - serum albumin precursor - bovine| 36.2 5.8 72.93
1 * - gi|229552|prf||754920A - albumin [Bos taurus]| 30.3 5.8 69.46
1 * - gi|113582|sp|P14639|ALBU_SHEEP - SERUM ALBUMIN PRECURSOR 15.7 5.8 72.85

Header	*Description*
Rank	Candidate proteins are ranked by the probability that the candidate protein is the sample protein.
Confidence	The statistical probability is presented as a confidence value. A value of 99 means 99% statistical probability (p=0.01).
Protein Information	The database accession number and the name of the proposed protein.
Coverage	Is defined as the ratio(%) of the protein sequence covered by the matched peptides.
pI	The isoelectric point of the proposed protein.
kDa	The molecular weight (kDa) of the proposed protein.

2 To view detailed information, select one of the protein candidates and select the tab **Protein detail**. Matched peptide masses are listed with error values and peptide sequences. Below the peptide list, there is a box with the unmatched masses.


Identification result
Protein list
Protein detail
Protein sequence
Selected protein
Rank: 1 Confidence: 0.99 Coverage: 37.9 pI: 5.8 Mass: 72.99 kDa Matched peptides: 23 Measured peptides: 40
Protein information: gi|2190337|emb|CAA41735.1 - (X58989) serum albumin [Bos taurus]
Matched peptides
Meas. mass Ave/Isotopic Comp. mass Error Res. start Res. end M. cut Peptide sequence
926.478 Mono 926.486 -0.008 161 167 0 YLYEIAR
973.477 Mono 973.451 0.027 37 44 0 DLGEEHFK
1081.440 Mono 1081.501 -0.062 123 130 0 NECFLSHK(1) +C7H7N@C
1115.335 Mono 1115.471 -0.135 413 420 0 QNCDQFEK(1) +C7H7N@C
1154.479 Mono 1154.543 -0.064 588 597 0 EACFAVEGPK(1) +C7H7N@C
1162.515 Mono 1162.623 -0.109 66 75 0 LVNELTEFAK
1233.617 Mono 1233.563 0.053 499 507 0 CCTESLVNR(2) +C7H7N@C
1248.585 Mono 1248.614 -0.029 35 44 1 FKDLGEEHFK
1282.660 Mono 1282.703 -0.043 361 371 0 HPEYAVSVLLR

3 To view a coverage graph, select the menu command **View:Coverage**.



Term	*Description*
Coverage	Is presented in the **Protein detail** window as the number of measured and matched peptides. The coverage is also described in the **Coverage** graph. The matched peptides are marked in the order of residue number to describe where in the sequence matching occurs.

4 The amino acid sequence of the proposed protein is shown when the tab **Protein sequence** is selected.

7.3 Manual evaluation of MS results

If only an Acquisition method has been applied previously to a sample, it is possible to perform additional analysis now. One reason for performing additional analysis may be to use alternative methods to improve the results. Only the first spectrum of a sample is automatically evaluated. So, additional spectra have to be evaluated manually.

1 In the **Evaluation** module, select the **Results** tab and **File:Open:Result**. Browse for the requested sample. Select it, and click on **Open**.The selected sample is shown in the workspace.

2 Double-click on the requested sample or use the “plus” sign. The tree structure shows for example that a spectrum has been acquired using an Acquisition method.

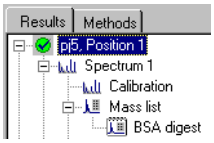
7.3.1 Inserting of a Spectrum processing method

1 To apply a Spectrum processing method to the spectrum, click on the spectrum icon, and select **Insert:Spectrum processing method**. In the **Open method** dialogue, browse and select a method, and click **Open.**

2 An alternative procedure is to use drag-and-drop from the **Favourite** window and apply it to the spectrum icon. Inserting is also possible with the right mouse button command **Insert:Spectrum processing method.**

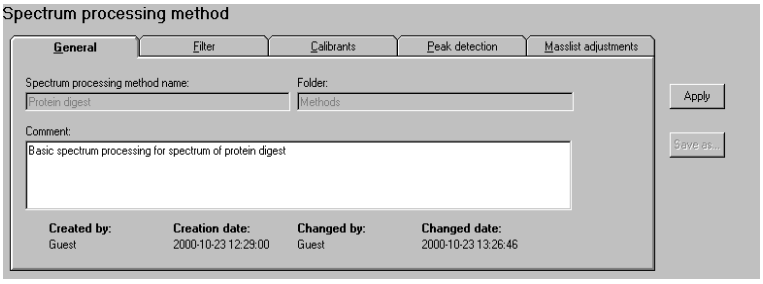
3 The spectrum will be analysed with the selected Spectrum processing method and a Mass list will be created within the spectrum structure.

7.3.2 Editing the Spectrum processing method

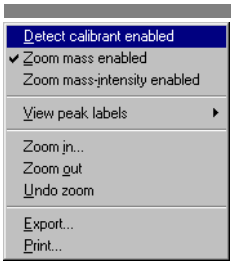


1 Select in the workspace the Spectrum processing method icon.

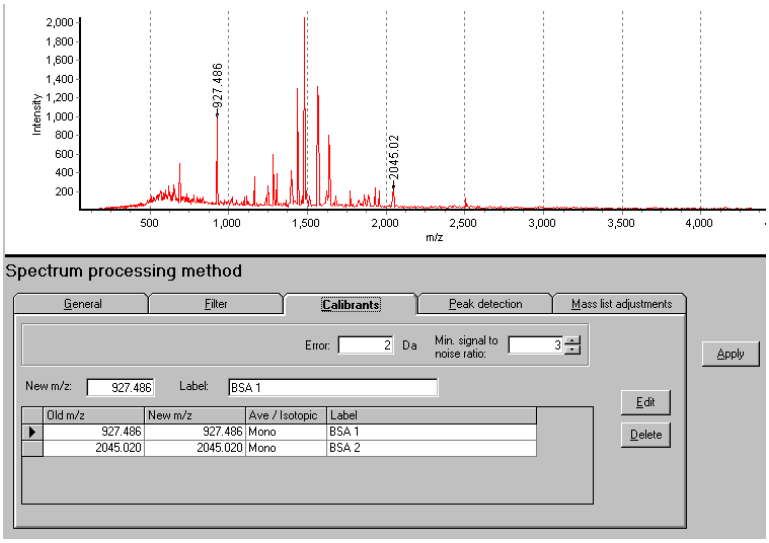
2 The Spectrum processing method will be opened in a window with tabs below the spectrum. The parameters are the same as in the Spectrum processing method described in Section 5.6 .



3 To apply a new calibration or reprocess a spectrum with edited parameters in the Spectrum processing method and create a new Mass list, select one of the tabs. Edit or enter new parameter values and click on **Apply.**

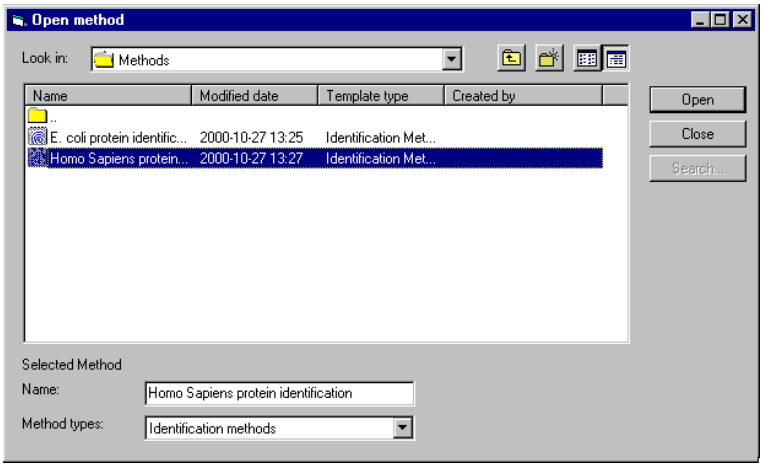


4 When the **Calibrants** tab is selected, the spectrum with used calibrants is displayed. To add a calibrant, select the right mouse button command **Detect calibrant enabled**, select a peak with the mouse cursor, enter m/z values and label and click **Edit**. Max. 4 calibrants are used.



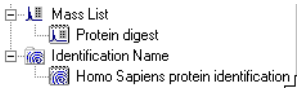
7.3.3 Inserting an Identification method

1 To create a protein identification from the mass list, click on the spectrum icon and select **Insert:Identification method**. Browse and select an Identification method and click **Open**.



2 An alternative, is to select the requested identification method in the **Favourites** window, and drag-and-drop it on the spectrum icon.

3 The mass list will be used for database search according to the selected Identification method. An identification result will be created in the identification substructure within the spectrum structure.



4 To obtain additional identification results, it is possible to add several identification methods to a mass list.

8 PSD investigations

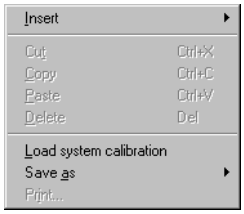
8.1 Introduction

When the protocol peptide mass fingerprinting has failed to identify a protein, it is possible to obtain additional data by PSD investigations using the same sample slide.

PSD investigations can also be used in studies of individual peptides to obtain parts of the amino acid sequences.

8.2 Selecting a peptide in a mass spectrum

1 In the **Evaluation** module, open the spectrum result of the investigation where identification failed. Choose one of the peptides for PSD investigation.



2 Analyse the sample in reflectron mode with internal calibrants. Click on the Calibration icon and use the right mouse button command **Save as:System calibration**. Note the exact mass value for the peptide to be studied.

8.3 Creating a PSD Acquisition method

1 In the **Control** module, select the tab **Methods** and **File:New:Method**. Select the Acquisition method template: PSD. Enter a new name and click **New.**This method includes preset values for ^{ile7}Angiotensin III and needs to be changed to suite the peptide being analysed. To edit the method, select the method in the workspace.

2 In the Instrument method editor, select **Detection mode** PSD.

3 Enter the mass value of the selected peptide in the **Ion gate mass** box.

4 Set a **Width** of m/dm ≤ 100 (see Section 5.5.2). Select **Ion polarity** Positive, **Acceleration potential** 20 kV, **Mass range** low. Select **Pulsed extraction** and enter the mass of the selected peptide in the **Focus mass:** box.

5 Leave the **Control parameters** for tuning empty. Under **Acquisition parameters**, select **No. of shots** and **No. of spectra** (generally >100 shots per spectra).

6 Save the Acquisition method and add it to **Favourites**.

8.4 Creating a PSD run list

1 Select the tab **Run lists** in the **Control** module and **File:New:Run list**.

2 Select an empty run list as template, enter a new name, and click **New**.

3 For the selected position, insert a Method setup and apply the created PSD Acquisition method to it. Drag-and-drop from the **Favourite** window, or use **Insert:Acquisition method.**

4 Save the run list.

8.5 Analysing a PSD sample

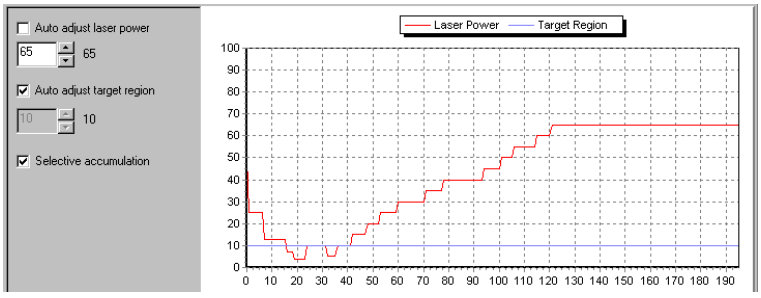
1 Insert the sample slide into the instrument.

2 Open the created run list. Select the run list in the work space.

3 Select **Process:Submit Run list**.

4 Select **Process:Start**.

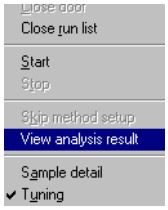
5 To open the tuning window, select **View:Tuning**.



6 Manually adjust the laser power and target region until the peak of the selected peptide is detected in the mass spectrum. Enter values in the white boxes. The current values will be shown at the right.

7 Slowly increase the laser power until PSD fragments are detected.

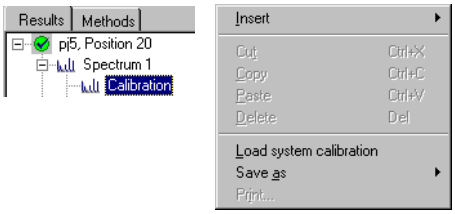
8 When the specified number of spectra has been acquired and the sample icon has turned green, click on the sample icon and use the right mouse button command **View analysis result**.



8.6 Spectrum processing of a PSD spectrum

1 In the Evaluation module, apply a Spectrum processing method to the spectrum. Click on the spectrum icon and select **Insert:Spectrum processing method.** Select e.g. the template PSD.

2 Click on the Calibration icon in the workspace and select the right mouse button command **Load system calibration.**



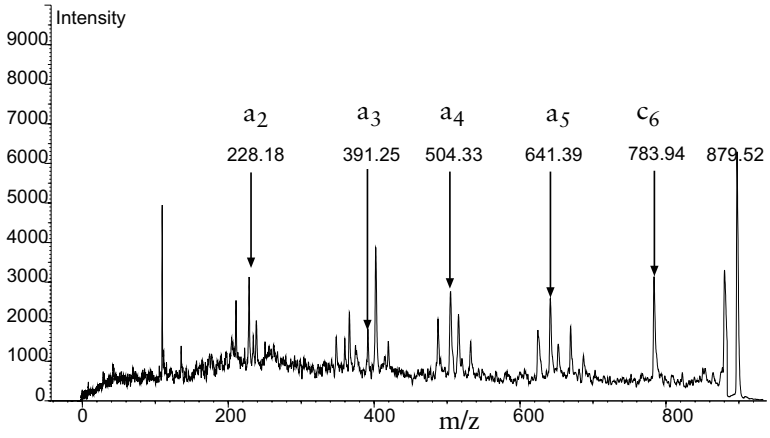


Fig 8-1. Example of a MS spectrum of ^{ile7}Angiotensin III, using PSD mode.

3 The created Mass list can be used as input values to database search.

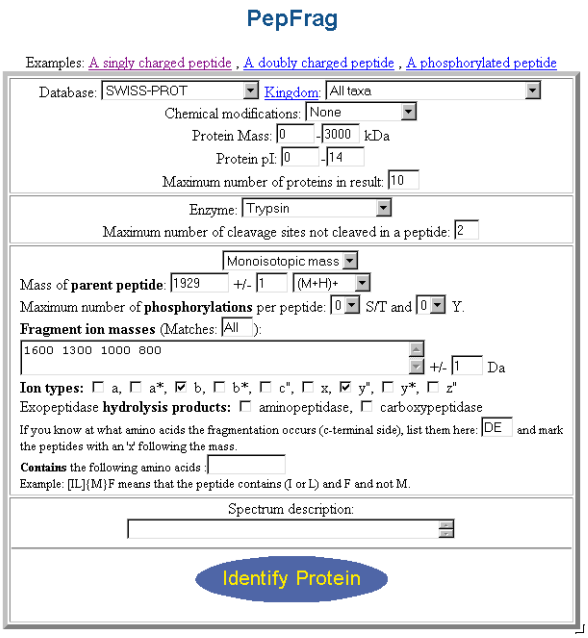
4 When the Mass list is active, select the right mouse button command **Copy m/z to clipboard.**

8.7 Database searching using PepFrag

Use the Internet site PepFrag of ProteoMetrics LCC (www.proteometrics.com).

1 Select relevant options and enter the mass value of the precursor ion and paste the masses of its fragments.
If known, select which type of fragments they are.

2 Click on **Identify Protein.**


PepFrag
Examples: A singly charged peptide , A doubly charged peptide , A phosphorylated peptide
Database: SWISS-PROT
Kingdom: All taxa
Chemical modifications: None
Protein Mass: 0 - 3000 kDa
Protein pI: 0 - 14
Maximum number of proteins in result: 10
Enzyme: Trypsin
Maximum number of cleavage sites not cleaved in a peptide: 2
Monoisotopic mass
Mass of parent peptide: 1929 +/- 1 (M+H)+
Maximum number of phosphorylations per peptide: 0 S/T and 0 Y.
Fragment ion masses (Matches: All):
1600 1300 1000 800
+/- 1 Da
Ion types: a, a*, b, b*, c", x, y°, y*, z"
Exopeptidase hydrolysis products: aminopeptidase, carboxypeptidase
If you know at what amino acids the fragmentation occurs (c-terminal side), list them here: DE and mark the peptides with an 'x' following the mass.
Contains the following amino acids :
Example: [IL]{M}F means that the peptide contains (I or L) and F and not M.
Spectrum description:
Identify Protein

3 The obtained result describes a proposed identity and amino acid sequence.

gi|123636|sp|P12398|HS77_YEAST HEAT SHOCK PROTEIN SSC1, MITOCH
mass = 70627.9 Da, pI = 5.5

PPAPKGVPQIEVTFDIDADGIINVSARDKATN mass = 2329.6

830.00 +/- 2.00 Da: **y"8** (829.98 Da)
1015.90 +/- 2.00 Da: **y"10** (1016.14 Da)
1243.80 +/- 2.00 Da: **y"12** (1244.39 Da)
1705.70 +/- 2.00 Da: **y"16** (1706.89 Da)

9 MS analysis of full-length proteins

9.1 Introduction

The Ettan MALDI-ToF can also be used for analysis of full-length proteins. Obtained mass spectra will give information about the molecular weight of a protein and the purity of the protein sample.

9.2 Creating a full-length protein Acquisition method

1 In the **Control** module, select **File:New:Method** and select the template "Full length protein", enter a new name, and click **New.** Select the method in the workspace.

2 In the Acquisition method editor, select **Detection mode** Linear, **Ion polarity** Positive and **Acceleration potential** 20 kV. Select **Mass range** High, and check **Pulsed extraction.** In the **Focus mass box,** enter e.g. 7000 (dependent on Mw and may need to be optimized). Max. value is 10 000.

3 Select auto adjust of target and laser power and enter tuning start 10-30 for **Target region** and 20 for **Laser power.**

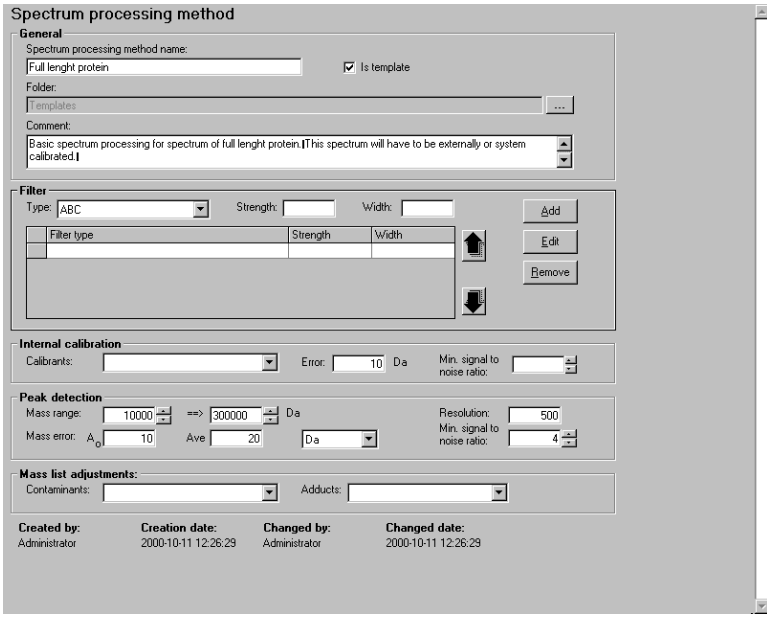
4 Enter **No. of shots** 100 and **No. of spectra** 5. Select **Selective accumulation** and enter a mass range (dependent on Mw) and intensities 2 and 50.

5 Save the method with the commands **File:Save** or **File:Save as** and add it to **Favourites.**

9.3 Creating a full-length protein Spectrum processing method

1 In the **Control** module, select **File:New:Method** and select the Spectrum processing method template: "Full length protein", enter a new name, and click **New**. Select the method in the workspace.

2 The method template can be used directly without any editing. The used calibration will be the system calibration of the instrument.



3 Save the method with **File:Save** and add it to the **Favourites** folder with **Tools:Add to favourites.**

9.4 Creating a run list

1 In the **Control** module, select **File:New:Run list**. Select a template and enter a new name in the **New run list name** box. Click **New.**

2 To insert Method setups to the positions, select **Insert:Method setup**. Apply the created Acquisition and Spectrum processing methods to the method setups by using drag-and-drop from the **Favourites** window or by **Insert:Acquisition method** and **Insert:Spectrum processing method**.

9.5 Analysing samples

The analysis is performed in the same way as for peptide mass fingerprinting, see Chapter 6 .

9.6 Evaluating results

1 In the **Evaluation** module, select **File:Open:Result**. Browse for the sample results and add them to the workspace.

2 Investigate the obtained spectrum by clicking on the spectrum icon. The calibration used in this case will be the system calibration or external calibration (see Section 4.5.2), because internal calibration is more difficult to utilize.

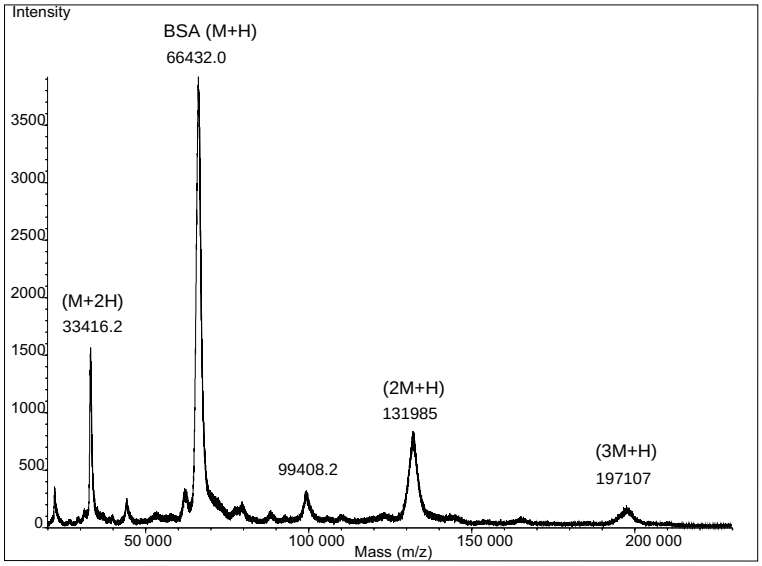


Fig 9-1. Example of a MALDI-TOF MS spectrum of bovine serum albumin.

3 In MALDI-TOF MS analysis of full-length proteins, different peaks are usually obtained. The first peak can be at half the m/z of the protein due to a double charge. The second peak indicates the molecular weight of the protein. The third and fourth peak are aggregates of the protein.

10 Practical examples

10.1 Mass determination of a peptide (hACTH fragment 18-36)

10.1.1 Sample preparation

A solution consisting of 20 pmol hACTH per µl in 50% Acetonitrile (ACN), 0.5% TFA was prepared. 2 µl of the material was mixed with 2 µl matrix solution (a saturated solution of α-cyan-4-hydroxy cinnamic acid in 50% ACN, 0.5%TFA) and 0.3 µl was directly spotted on to the sample slide. Thus, approximately 3 pmol hACTH was crystallised onto the sample target. After the material had dried (dried droplet method) it was analysed in the mass spectrometer.

10.1.2 MALDI-TOF MS analysis

The main instrument parameters were:

- 20kV, Reflectron mode
- Ion polarity: positive
- Low mass range selected
- Pulsed extraction: On (Focus mass: 2500)
- Low mass rejection: On(Mass limit: 1000)
- 1 shot / second laser mode
- Spectra consisting of 25 shots each were acquired (10 spectra)
- System calibration was used

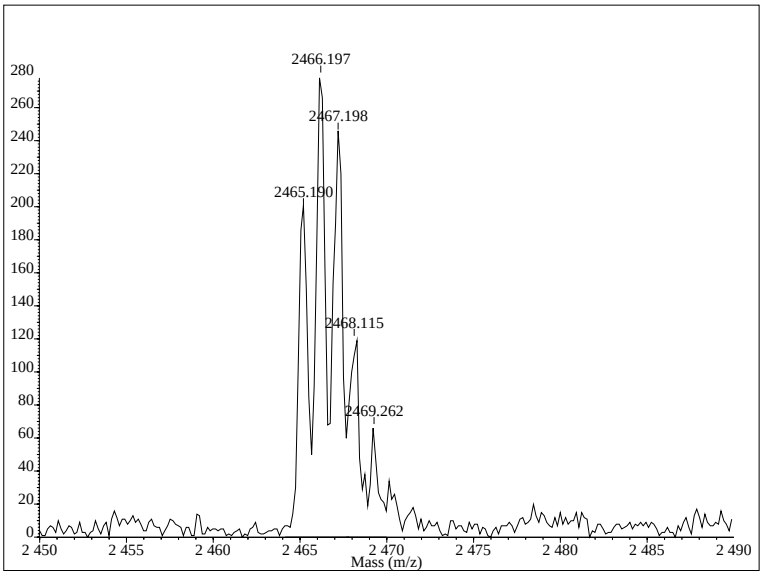


Fig 10-1. Mass spectrum of hACTH fragment 18-36

In the spectrum the isotopic cluster of hACTH at m/z 2465 is clearly detected. The isotopic cluster is formed due to the natural incidence of ^{13}C in the environment. The first peak seen in the cluster is the monoisotopic peak consisting of only the most common basic element isotopes. The area under the peaks shows the chance of having a molecule with 0, 1, 2, etc. ^{13}C atoms. The difference between the isotopes also shows the charge state of the ion. If the m/z difference is 1 amu (as in the spectrum shown) the charge is +1, but if the charge had been +2 the m/z difference would have been 0.5. The precision of the instrument and to some extent the mass accuracy is also seen in the isotopic cluster. Since we know that the charge is +1 the difference in mass should be 1.00 amu (^{13}C=13.00335). In the spectrum it is seen that the difference between the first (monoisotope), the second, and the third peak is 1 ± 0.01 amu, which is as expected. The resolution of this particular spectrum is in the range of 4000 FWHM.

10.2 Mass determination of full-length BSA

10.2.1 Sample preparation

A solution consisting of 60 pmol BSA per µl in water was prepared. 2 µl of the material was mixed with 8 µl matrix solution (a saturated solution of sinapic acid in 50% ACN, 0.9% TFA) and 0.3 µl was directly spotted on to the sample slide. Thus, approximately 3.5 pmol BSA was crystallised onto the sample target. After the material had dried (dried droplet method), it was analysed in the mass spectrometer.

10.2.2 MALDI-TOF MS analysis

The main instrument parameters were:

- 20kV, Linear mode
- High mass range selected
- Pulsed extraction: On
- Low mass rejection: Off
- 8 shot / second laser mode
- One spectra consisting was acquired (136 shots)
- The system calibration was used

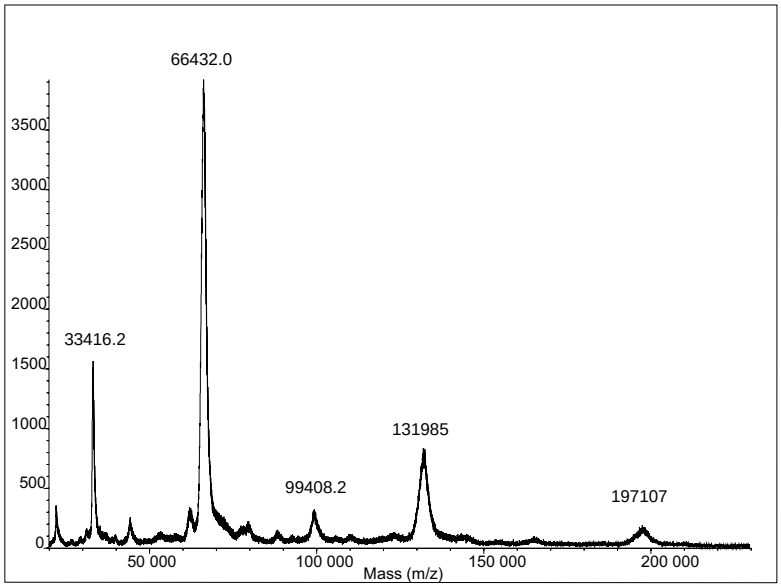


Fig 10-2. Mass spectrum of BSA

In the spectrum the major peak of BSA is clearly seen at m/z 66 000. A double charged ion of BSA is detected at m/z 33 000. These double charge ions are more readily detected using sinapic acid as the matrix. Interestingly, multimers of BSA are detected in the spectrum. The double mass is detected at 132 000 and even the triple mass aggregate is detected at 197 000. Furthermore, additional peaks are also detected in the spectrum (45 000, and 99 000, etc.). The spectrum shown has not been filtered and the raw data is displayed.

10.3 Peptide mass fingerprinting: Identification of a protein in solution

This method is of particular use for the identification of proteins from LC-purification or similar. The final amount used for the identification in the Ettan MALDI-ToF was in the order of 200 fmol.

10.3.1 Digestion and sample preparation

1 The lyophilised material (2.4 mg) was dissolved in 800 µl of a buffer solution consisting of 8M urea, 50 mM, Tris-HCl pH 8.0, and 50 mM DTT.

2 After incubation at 30°C for 30 minutes, 10 µl re-distilled 4-vinyl pyridine was added (this was done to modify the cys-residues).

3 After 1 hour of incubation at 30°C, the sample was desalted using a NAP-10 column (equilibrated with 100 mM NH_4HCO_3 pH 8.8) and eluted in 1.2 ml.

4 The sample was then supplemented with trypsin (Promega #V5111) to a final ratio of 1 µg trypsin/100 µg protein.

5 The digestion mixture was incubated for 6 hrs at 30°C and the reaction was stopped by the addition of TFA to a final concentration of 1%.

6 The digest was stored at -20°C, and diluted in 50% ACN, 0.5%TFA before analysis.

For the MALDI-TOF MS analysis, 1 µl of the diluted digest from step 6 was mixed with 1 µl matrix solution (a saturated solution of α-cyan-4-hydroxy cinnamic acid in 50% ACN, 0.5%TFA). Directly after mixing, 0.5 µl was spotted into the sample slide (dried droplet method), and let to air dry. Approximately 250 fmol of the material was applied onto the target for analysis.

10.3.2 MALDI-TOF MS analysis

The main instrument parameters were:

- 20kV, reflectron mode
- Ion polarity: positive
- Pulsed extraction: On (Focus mass 2 000)
- Low mass rejection: On (500)
- 8 shot / second laser mode

- Auto tuning of laser power and target
- Selective accumulation (800-3 000)
- One spectrum was acquired (250 shots)
- Internal calibration

The spectrum was calibrated using peptides included in the matrix solution. Approximately 100 fmol of ^{ile7}AngIII (M+H 897.52, monoisotopic) and hACTH 18-39 (M+H 2465.19, monoisotopic).

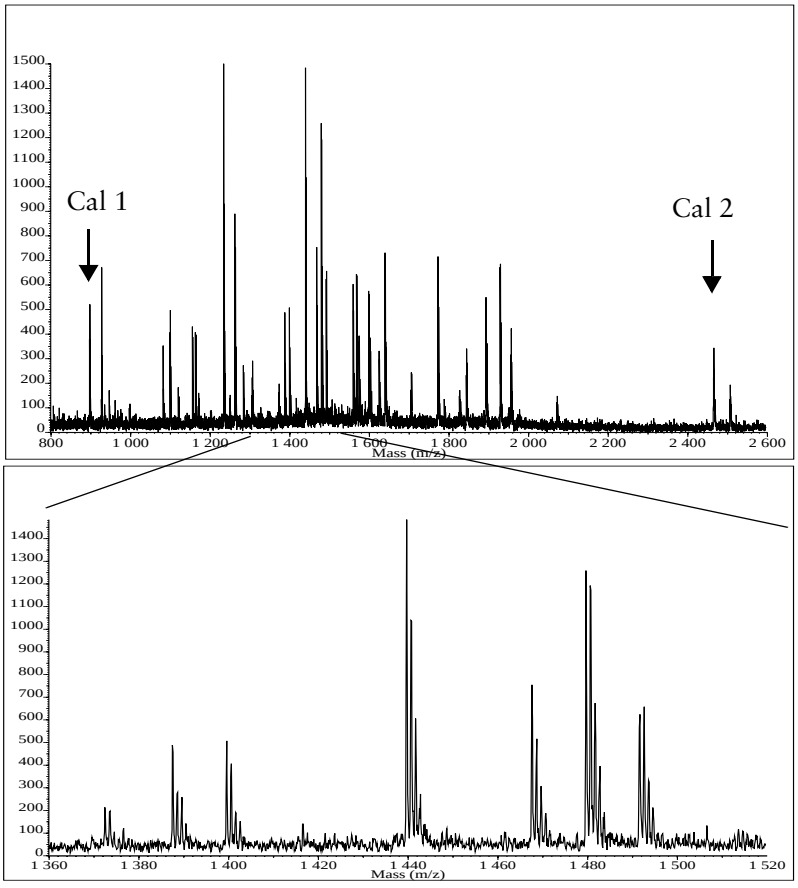


Fig 10-3. Mass spectrum of protein digest

After calibration the mass of the monoisotopic peaks in the spectrum (between 850-2600 m/z) were determined using the Spectrum

processing method in the Ettan MALDI Software. A mass list of the monoisotopic peptide fingerprint was thus generated.

897.530 Cal 1	927.490	935.390	946.470	960.360	968.450
997.540	1082.450	1099.400	1120.540	1155.520	1163.610
1171.510	1234.550	1249.610	1262.580	1283.720	1305.710
1372.640	1387.680	1399.700	1439.820	1467.750	1479.810
1491.760	1559.700	1567.790	1574.620	1598.720	1602.720
1624.840	1639.990	1704.790	1772.840	1788.890	1826.720
1843.780	1893.740	1928.930	1955.990	2071.960	2465.190 Cal 2
2506.260					

Table 10-1. Mass list

10.3.3 Protein database search

The mass list was then submitted to the Identification method in the Ettan MALDI Software and a Protein database was searched.

The indata was: Trypsin as cleaving enzyme, maximum 1 missed cleavage, 4-vinyl-pyridine (Cys) as complete modification, Methionine as partial modification, the masses were entered as mono isotopic masses and the mass tolerance of ± 100 ppm was set.

10.3.4 Identification result

The top candidate Bovine Serum Albumin, gi1351907, P02769) was scored with a high probability (99%). The result showed that 33 of the 43 submitted peptides were matched to belong to the top candidate and the sequence coverage was found to be 54%, which is a high number for a MALDI spectrum.

From the results it was shown that not all peptides were scored as being part of the candidate. The peaks at 897.53 and 2465.19 were the calibrant peptides and was not included in the search. Some of the peaks were from the chemical background (keratin, etc.). 1099.4 is one such peak. The peaks at m/z 968.45, 997.5, 1372.6 could be Na adducts of peptides 945.46, 974.5 and 1349.5, respectively.

10.3.5 Discussion

In this case, only one spectrum was collected. It is then advisable to collect more shots than usual (in this case 250 compared to normally 25-50 shots per spectra, or max up to 100 shots/spectrum, if the signals are expected to be low).

If several spectra have been acquired, start by looking at the peptides used as calibrants and go through the different spectra to find the one with the best resolution of the isotopes. This is in order to get the best possible calibration. However, the peptides of interest for the identification should then also be mass determined in this spectrum.

If the instrument parameter "low mass rejection" has been set to a value close to the used low mass calibration peptide, in our case 897, there is a risk of affecting calibration of the spectra due to interference of the time of flight for this ion. This will show itself as a mass accuracy problem in the search results with the peptide masses clustered but with a too high mass error.

10.4 Peptide mass fingerprinting: Identification of a protein detected in a 2D-electrophoresis gel

10.4.1 Starting material and electrophoresis

This method is of particular use for the identification of proteins from polyacrylamide based gel. The starting material was a saline buffer used to flush through the human lung in the clinic. The sample was desalted using an anion exchange column and approximately 3 mg was loaded onto a narrow range IPG-strip (pH 5.0-6.0) using sample cup at the anodic end. The gel was stained using coomassie brilliant blue.

10.4.2 Digestion and sample preparation

1 The spot of interest was excised from the gel in an approximately 2 mm diameter gel plug.

2 The gel plug was washed with 150 µl of 0.2 M ammonium bicarbonate (AMBIC) / 50% ACN at 30°C for 1-2 hours.

3 The washing step was repeated with 0.05 M AMBIC / 50% ACN.

4 The gel plug was dried to completion in a speed vac.

5 The dried gel plug was reconstituted using 5 µl of Trypsin (0.1 µg / µl, Promega modified trypsin).

6 To start the digestion, 20 µl of 0.05 M AMBIC was added to cover the plug, and incubated at 30°C over night.

7 After incubation, the remaining supernatant was transferred to a new tube.
100 µl of a solution containing 50% ACN / 0.45% TFA was added to the gel plug in order to extract the peptides from the gel plug. After incubation at 30°C for one hour, the step was repeated.

8 The pooled supernatant (approximately 200 µl) was dried in a speed vac and the pellet was stored at -20°C until use.

For the MALDI-TOF MS analysis, 1 µl of the digest from step 8 (reconstituted in 5 µl 0.1% TFA) was mixed with 1 µl matrix solution (a saturated solution of α-cyan-4-hydroxy cinnamic acid in 50% ACN, 0.5% TFA). Directly after mixing 0.5 µl was spotted into the sample slide (dried droplet method), and let to air dry. The final amount used for the identification in the Ettan MALDI-ToF was estimated to be approximately 100 fmol on target.

10.4.3 MALDI-TOF MS analysis

The main instrument parameters were:

- 20kV, reflectron mode
- Ion polarity: positive
- Pulsed extraction: On (Focus mass 2 000)
- Low mass rejection: On (500)
- 1 shot / second laser mode
- Auto tuning of laser power and target
- One spectrum was acquired (100 shots)
- Internal calibration

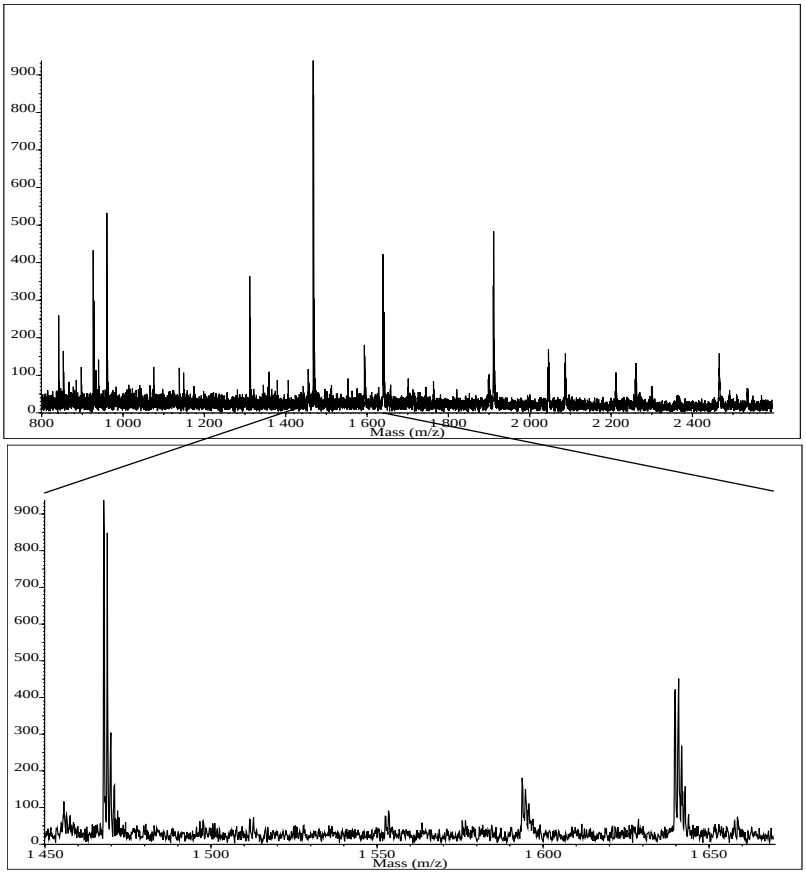


Fig 10-4. Mass spectrum of in-gel digestive protein

The spectrum was calibrated using peptides included in the matrix solution. Approximately 100 fmol of ^{ile7}AngIII (M+H 897.52, monoisotopic) and hACTH 18-39 (M+H 2465.19, monoisotopic)

After calibration the mass of the monoisotopic peaks in the spectrum (between 850-2600 m/z) were determined with the Spectrum processing method. A mass list of the monoisotopic peptide fingerprint was thus generated.

926.512	932.532	939.502	959.592	1310.762	1466.782
1593.720	1638.762	1656.682	1897.902	1909.902	2044.052
2085.812	2259.062				

Table 10-2. Mass list

10.4.4 Protein database search

The mass list was then submitted to the Identification method in the Ettan MALDI Software and a Protein database was searched.

The search parameters were: "all taxa" (i.e. the entire database volume was searched), open windows for pI and Mw, monoisotopic masses with a maximum error of 100 ppm, 1 missed cut allowed, Trypsin cleavage, Cys modified with acetamide (Iodoacetamide incubation between the first and second dimension separation of the 2D-GE), allowed for partial Met oxidation (+16 amu).

10.4.5 Identification result

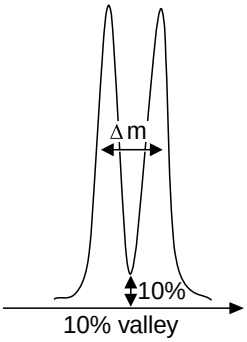
The top candidate was scored with a high probability (99%) and in the detailed search results view it was found that the candidate had reasonable sequence coverage (23%) and a high mass accuracy, thus the result is of high confidence. The result was human serum albumin, gi6013427. Peaks that showed poor S/N or bad resolution were not included into the mass list due to the settings in the Spectrum processing method.

The protein identity scored was in good agreement with the 2D-gel in terms of pI and Mw, thus taken together the search result seems valid. Of the 14 submitted masses 13 were scored to the candidate with a similar mass accuracy as in the previous experiment.

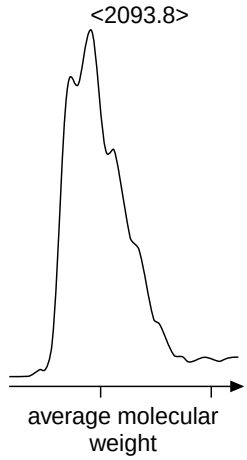
10.4.6 Discussion

The peptide not matched (1593.720) has not been assigned to any known adducts, and remains “orphan”. Low intensity peaks are detected around 840, and 2210 which are trypsin auto digestion peaks. The peak at 2465 is the calibrants, and the other calibration peak at 897 is also present but was “suppressed” by the other peptides in the spectrum. The phenomenon of suppression is unclear but it is evident that peptides can “suppress” the signal from other peptides in MALDI-Of MS. The samples from 2D-GE and other electrophoresis also has a higher "chemical noise" due to the steps performed on them during digestion, extraction, etc.

11 Glossary



Term (Abbreviation)	*Description*
10% valley	For defining resolution. Two peaks of equal height in a mass spectrum at masses m and m-Δm are separated by a valley that at its lowest point is just 10% of the height of either peak.
ablation step	Molecular components of a solid are put into a gas phase and ionized.
adaptive background correction (ABC)	ABC filters are usually also applied to low resolution data to enhance the high-frequency signal and remove the low frequency background: $I'_j = I_j - d/(2n+1)\sum I_{j+k}$ $k = -n,\ldots,n$, where $0 < d < 1$, n is the width of the filter, I_{j+k} is the intensity in channel j+k before the filter has been applied to the data, and I'_j is the intensity in channel j after the filter has been applied to the data. By applying an ABC filter in combination with a smoothing filter the quality of peaks can be improved.
adduct peak	Results from the photochemical breakdown of the matrix into more reactive species, which can add to the polypeptide. Can also result from salt ions, Na^+ etc., that are embedded in the matrix.
average molecular weight	The mass of a molecule of a given empirical formula calculated using the average atomic weights for each element. An average mass is obtained in MALDI-TOF-MS when a peak is not isotopically resolved.
background subtraction	The process in which the background (chemical noise and detector noise) is subtracted, thus leaving the peaks above the noise at the base level.

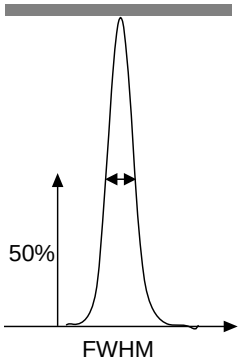


Term (Abbreviation)	*Description*
base peak	The most intense peak in a mass spectrum. A mass spectrum is usually normalized so that this peak has an intensity of 100%. See also parent peak.
calibrant	A compound used for the calibration of an instrument.
calibration	A process where well-known masses are assigned to certain peaks. The purpose is to improve the mass accuracy of an MS instrument. For ESI-TOF, the calibrant is introduced into the chamber via a second electrospray needle. For MALDI-TOF-MS, the calibrant is either mixed with the sample (internal calibration) or run as a separate sample (external calibration).
centroided mass peak	The centroided mass peak is located at the weighted centre of mass of the profile peak.
collision induced dissociation (CID)	A process whereby a mass-selected ion is excited and caused to fragment by collision with a target gas. Used in some ESI and MALDI instruments. The fragment ions need to be charged to be mass separated, so many of the MALDI fragments may not be detected since they are neutral.
C-terminal	The carboxyl residue at the end of a polypeptide chain. Proper nomenclature for indicating product ions containing a C-terminal is v, w, x, y, and z.
daughter ion	See fragment ion
desorption ionization (DI)	An ionization method used to ionize non-volatile solid samples by impact of energetic particles or photon beams.
digestion	Cleavage by proteolytic enzymes such as trypsin and chymotrypsin.
dried droplet method	Sample preparation method. A droplet of a protein-matrix solution is dried. When the solvent has evaporated, the sample can be loaded into the MALDI mass spectrometer.

Term (Abbreviation)	*Description*
electrospray (ES)	Comprises the formation of electrically charged droplets created from a flowing liquid sample, the extraction of gas phase ions from these droplets under a high electric field, and transportation of the ions into a vacuum suitable for MS.
electrospray ionization (ESI)	An ionization method that allows production of molecular ions directly from samples in solution. A sample solution is sprayed through a needle, which is kept at a potential (typically 3.5 to 6 kV). The voltage on the needle causes the formed droplets to be charged. As the solvent evaporates, the droplets disappear leaving highly charged analyte molecules. The resultant ions often carry multiple charges, which reduces their mass-to-charge ratio compared to a singly charged species. It is a continuous method suitable for interfacing with HPLC or capillary electrophoresis. It is also a very sensitive method well suited for the analysis of small amounts of large biological molecules such as peptides and proteins that are difficult to vaporize or ionize, especially thermally labile compounds. Generates ions carrying many charges.
external calibration	Calibration where a set of calibration constants that have been measured in a previous experiment is applied. The calibrant is run as a separate sample.

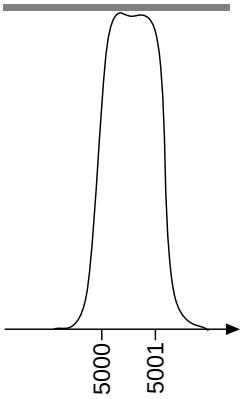
Term (Abbreviation)	*Description*
FASTA format	A format of sequence databases. A sequence in FASTA format begins with a single-line description, followed by lines of sequence data. The description line is distinguished from the sequence data by a greater-than (">") symbol in the first column. It is recommended that all lines of text be shorter than 80 characters in length. An example sequence in FASTA format is: >gi|532319|pir|TVFV2E|TVFV2E envelope protein ELRLRYCAPAGFALLKCNDADYDGFKTNCSNVSV VHCTNLMNTTVTTGLLLNGSYSENRT QIWQKHRTSNDSALILLNKHYNLTVTCKRPGNKT VLPVTIMAGLVFHSQKYNLRLRQAWC HFPSNWKGAWKEVKEEIVNLPKERYRGTNDPKR IFFQRQWGDPETANLWFNCHGEFFYCK MDWFLNYLNNLTVDADHNECKNTSGTKSGNKR APGPCVQRTYVACHIRSVIIWLETISKK TYAPPREGHLECTSTVTGMTVELNYIPKNRTNVTL SPQIESIWAAELDRYKLVEITPIGF APTEVRRYTGGHERQKRVPFVXXXXXXXXXXXXXX XXXXXXXXVQSQHLLAGILQQQKNL LAAVEAQQQMLKLTIWGVK

Term (Abbreviation)	*Description*
filters	Filters are commonly applied to time-of-flight mass spectra to improve the appearance of a spectrum and to extract peaks from poorly resolved signals. The general form for one type of filters applied to mass spectra is: $I'_j = c\sum I_{j+k}F_k \;\; k = -n,\ldots,n,$ where c is a normalization constant ($c = 1/(\sum F_k)$ $k=-n,\ldots,n,$), n is the width of the filter, I_{j+k} is the intensity in channel j+k before the filter has been applied to the data, F_k is filter factor k, and I'_j is the intensity in channel j after the filter has been applied to the data. The filters that are useful for processing mass spectra do not shift the peaks and are therefore required to be symmetric ($F_i = F_{-i}$, $i = 0, 1,\ldots, n$); and of odd order ($n = 1, 3, 5,\ldots$).
fragment ion	A charged dissociation product of ionic fragmentation. The ion is generated by fragmentation of an ion and not generated by direct ionization of a neutral molecule. Used to be called daughter ion.
fragmentation	A physical process of dissociation of molecules into fragments in a mass spectrometer. The mass spectrum of fragments is unique for the molecule or ion. The fragments can be either ionic or neutral. Fragmentation can be used to provide sequence data for the identification of proteins and peptides.
fragmentation pattern	Set of reactions leading from the molecular ion to fragment ions. Molecules have distinctive fragmentation patterns that provide structural information to identify structural components.
fragmentation process	If the excess ionization energy in a molecular ion is greater than the energy required to break a chemical bond, the molecule can fragment. There are typically two types: direct cleavage (e.g. cleavage of a C-C bond) or rearrangement.



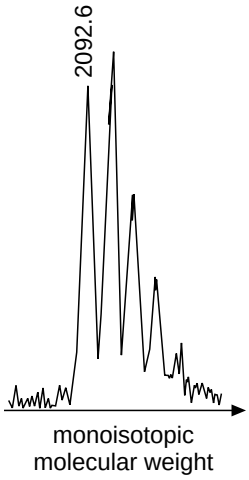
Term (Abbreviation)	*Description*
full length protein	An intact polypeptide chain, constituting a protein in its native or denatured state. One of the most common applications of mass analysis is where the protein is identified from its full length using a MALDI-TOF-MS.
full width at half height (FWHM)	A measure of the peak width at 50% of its maximum intensity.
high-pass filters	High-pass filters are usually applied to low resolution data to enhance the high-frequency signal and remove the low frequency background. An example of a simple high-pass filter (n=5) is F = {,-1,-1,-1,-2,1,F_0,1,-2,-1,-1,-1}. By applying a high-pass filter in combination with a smoothing filter the quality of peaks can be improved.
in-gel digestion	The second step in transferring the analyte from a 2D-gel to the MS analysis equipment. The protein in the gel is cleaved in a controlled way, usually with enzymes, although chemical cleavage can also be used. During the process, peptides are formed, which can easily be removed from the gel. The process also includes a number of washing and drying steps.
internal calibration	Calibration where known peaks are used in each spectrum to calculate a calibration for that particular data set. The calibrant is mixed with the sample. More accurate than external calibration.
ion detector	A detector that amplifies and converts ions to an electrical signal.
ion gate	A unit that electrically lets ions through to later stages of ion optics (open), or that can deflect the ions out of the way of the later stages of the mass spectrometer.
ion source	Device used to generate sample ions by MALDI or ESI, for example. In MALDI, the probe and the laser (and the extraction electrodes) are together referred to as the ion source.

Term (Abbreviation)	*Description*
ion transmission efficiency	Refers to the fraction of the ions produced in the source region that actually reaches the detector. Important measure of the sensitivity of the mass spectrometer.
ionization	The generation of an ion. A compound must be charged or ionized to be analysed by a mass spectrometer.
isotope	Atoms of the same element having different mass numbers due to differences in their number of neutrons.
isotope abundance	The relative amount in nature of certain atomic isotopes. Note that the atomic weight of an element is a weighted average of the naturally occurring isotopes. A high resolving mass spectrometer can separate the isotopes.
linear time-of-flight mass analyser	Consists of a long straight, empty flight tube with an ion source at one end and a detector at the other.
MALDI spectrum	A typical MALDI spectrum includes the molecular ion, some multiply-charged ions (only for heavy ions), and very few fragments. A MALDI spectrum results from a mass spectrometer utilizing a MALDI ion source.
mass accuracy	The difference between a measured value and the true mass of the substance.
mass analyser	Separates ions according to their *m/z* value. The ions enter the analyser either continuously (beam) or pulsed (using an ion gate or a pulsed ion source such as MALDI). Examples of mass analysers are: electrostatic-magnetic, ion trap, quadrupole, time-of-flight.
mass range	The difference between the upper limit and the lower limit of a measurement. For example, the range of mass-to-charge ratio (m/z) that a mass analyser can analyse. Can be <2 000 (low) to > 500 000 (high).



Term (Abbreviation)	*Description*
mass resolution	The degree of separation between two adjacent peaks in a mass spectrum. The resolution is a measure that can be used to determine if two adjacent peaks will overlap or not. For example, FWHM resolution of 5000 means that two peaks at mass 5000 and 5001 will just join with no valley in-between.
mass spectrometer	An instrument that uses the difference in mass-to-charge ratio (*m/z*) of ionized atoms or molecules to separate them from each other. All mass spectrometers consist of three main parts: an ion source, a mass analyser, and an ion detector. Used for determining chemical and structural information and for quantification of atoms and molecules.
mass spectrometry (MS)	A technique for analysing the molecular weight of molecules based upon the motion of a charged particle in an electric or magnetic field.
mass spectrum	A plot of ion abundance (Y-axis) vs. mass-to-charge ratio (X-axis). It contains information on exact mass (i.e. elemental composition), fragmentation pattern (i.e. structure and "fingerprint") and isotope abundance (i.e. presence and number of certain elements).
mass-to-charge ratio (*m/z*)	A quantity formed by dividing the mass of an ion (in Da units) by the number of charges carried by the ion.
matrix	In MALDI, referred to as a light-absorbing compound that forms crystals that incorporate proteins, eject the proteins intact, and ionize them in the same process.
matrix-assisted laser desorption/ ionization (MALDI)	The analyte is dissolved in a solution with an excess of a matrix containing a chromophore that absorbs at the laser wavelength. The mixture is recrystallized and then vaporized in vacuum by a short flash of laser light. The ionized molecules can then be extracted into a mass spectrometer.

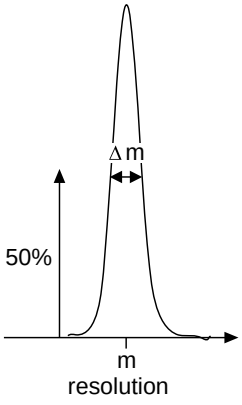
Term (Abbreviation)	*Description*
matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS)	Used to determine the molecular weight of peptides, proteins, oligonucleotides, and other compounds of biological origin as well as synthetic polymers. The analysis can be performed in linear mode (high mass, low resolution) up to a molecular weight of *m/z* 300 000 (in rare cases), or reflectron mode (lower mass, higher resolution) up to a molecular weight of 4 000.
metastable ion	An ion that decomposes into a charged product ion and a neutral species, or into two charged fragments, during its transmission through the mass spectrometer. Is normally a short-lived reaction intermediate or a vibrationally highly excited molecule.
method setup	A group of methods for collecting and analysing data. It consists of a method for the instrument followed by analyses and reports.
monoisotopic molecular weight	The mass of a molecule containing only the most abundant isotopes, calculated with exact atomic weights. In MALDI-TOF-MS, a monoisotopic mass is obtained in high-resolution mass measurement when the first isotopic peak of the distribution can be identified.
multiple-charged ion	Ion with more than a single charge and having correspondingly reduced mass-to-charge ratio.
normalization	All peaks are reported with peak heights relative to the highest peak height or area in the spectrum.
N-terminal	The amino residue at the end of a polypeptide chain. Proper nomenclature for indicating product ions containing an N-terminal is a, b, c, and d.
parent ion	See precursor ion.
parent peak	Refers to the peak of an ion that will be selected for fragmentation. The result of fragmenting the precursor ion is the fragment ions (several fragments from one precursor ion).



Term (Abbreviation)	*Description*
peak	The portion of a spectrum where a maximum can be found.
peak area	The area bounded by the peak and the base line. Can be calculated by integrating the abundances from the peak start to the peak end.
peak detection	The process of finding a *m/z* value from a peak in a mass spectrum. Commonly, the centroid method is used.
peak extraction	The process of finding all peaks in the mass spectrum (filtered according to specific parameters such as the threshold level). Compare with peak detection.
peak height	The distance between the peak maximum and the baseline.
peak resolution	The extent to which the peaks of two components overlap or are separated. Compare with FWHM.
peak value	The local maximum of the signal intensity within a small mass interval.
peak width	The width of a peak at a given height. Compare with FWHM. It is used to define resolution.
peptide-mass fingerprinting	The use of chemical or enzymatic agents to digest protein molecules according to the specificity of the agent, thus creating a unique set of peptide fragment molecular masses that can be detected via MS and used to search modified protein databases.
Pirani gauge	A thermal conductivity gauge, that measures the pressure of the gas by the resistance change caused by the density-dependent (thus pressure-dependent) cooling effect of the vacuum rest gas. Can be an instrument that measures very low pressures in gases, dependent on the conductivity of the gas.

Term (Abbreviation)	*Description*
post-source decay (PSD)	A method for obtaining sequence information about a single peptide observed in the mass spectrum. It describes the fragmentation processes that occurs in TOF in the drift region of the analyser. The PSD is used to fragment peptides within the MALDI-TOF-MS. The peptide is ionized at an increased laser power, thus giving it more internal energy. The bonds are broken apart giving rise to a product ion and a neutral fragment. Less fragmentation occurs than in CID.
post-translational modification (PTM)	A common name for several hundred types of modifications such as phosphorylation or glycosylation. Practically all protein modifications are associated with either an increase or a decrease in mass. Identification of a modification requires the specific detection of one particular modified peptide.
precursor ion	Any ion (including negatively and multiply-charged ions) that gives fragments. Used to be called parent ion.
probe	A sample slide used to introduce the sample into the mass spectrometer.
protein characterization	The identification of structural aspects of a protein. Includes amino acid sequence, molecular weight, three-dimensional structure, post-translational modifications and biologic activity of a particular protein.
protein sequencing	The determination of the order of amino acids in a particular protein or peptide. This determination can be achieved via mass spectrometry or chemical degradation techniques of either the N-terminal or the C-terminal of the protein or peptide.
proteome	The complete profile of proteins expressed in a given tissue, cell or biological system at a given time. A quantitative protein expression pattern of a genome under defined conditions.

Term (Abbreviation)	*Description*
proteome analysis	A systematic analysis comprising separation, identification, and quantification of many proteins from a sample at the same time.
proteomics	Systematic analysis of the protein expression of healthy and diseased tissues.
protocol	A detailed description of how to prepare and analyse the sample. Also called procedure.
pulsed mass analyser	Includes time-of-flight, ion cyclotron resonance, and quadrupole ion traps. An entire mass spectrum is collected from a single pulse of ions.
real time monitoring	A data acquisition method in which raw data are generated and displayed as the experiment is in progress.
reflectron	Improves resolution of a mass spectrometer. A series of rings or grids act as an ion mirror. Compensates for the spread in kinetic energies of the ions as they enter the drift region.
relative abundance (RA)	Normalization of a peak relative to base peak.
resolution	Refers to the separation of two ions where R=m/Δm. There are different definitions of the terms Δm, 10% valley, FWHM or peak width. For a single peak made up of singly charged ions at mass m in a mass spectrum, the resolution may be expressed as m/Δm where Δm is the width of the peak at a height that is a specified fraction of the maximum peak height. It is recommended that one of the three values 50%, 5% or 0.5% be used.
run list	A defined set of parameters that define the operating settings for a sequential list of samples. It allows the user to run the list of samples,. Each run list could contain one or several sample positions.



Term (Abbreviation)	*Description*
sample preparation	A crucial stage to achieve ionization. In MALDI, the sample is incorporated into the crystals of a second material, the matrix. There are several sample preparation methods that can be used for this incorporation, for example, the dried droplet method, thin layer method, slow crystallization method and seeded microcrystalline film method.
sample slide	Slide with numbered wells where the samples are put before they are introduced into the mass spectrometer. Also called probe or target plate.
seeded microcrystalline film method	A sample preparation method. First, a thin layer of small matrix crystals is formed on the sample slide. Then a droplet containing the analyte is placed on top of this layer. This is left to dry. The deposit is washed before the sample slide is inserted into the mass spectrometer. Is a direct replacement of the dried droplet method.
signal-to-noise ratio (S/N)	The ratio of the signal height and the noise height. An indication of the clarity of the data.
slow crystallization method	A sample preparation method. Here, large matrix crystals are grown. The analyte is added to a saturated matrix solution. Microcrystals are formed. The supernatant is removed and a slurry of crystals is made. The slurry is then applied to the sample slide, allowed to dry and inserted into the mass spectrometer. Can be used when the dried droplet method has failed.
smoothing filters	Smoothing filters are usually applied to noisy data to reduce the high-frequency noise associated with the signal, i.e. they are a type of low-pass filters. An example of a simple smoothing filter (n = 1) is $F = \{1,1,1\}$, $c = 1/3$. By applying few points wide smoothing filter the quality of peaks can be improved. However, if the width is increased the resolution deteriorates.
template	A pre-programmed method with default values. The user may have to add certain data (for example, sample volume). Templates may be edited and stored as user-defined methods.

Term (Abbreviation)	*Description*
thin layer method	A sample preparation method. Applying the matrix onto the substrate creates a very thin layer of matrix. A droplet of the analyte is then dried onto this layer. Contaminants can now be washed away before introducing the sample into the mass spectrometer. A variant of the dried droplet method.
threshold fluence	The lowest laser fluence at which protein ions start to appear in MALDI.
timed ion gate	Enables PSD by ion selection. See also ion gate.
time-lag focusing (TLF)	Improved resolution is obtained for a specified mass range by applying a controlled delay between ion formation and acceleration.
time-of-flight (TOF)	Separates ions in time as they travel down a flight tube.
time-of-flight mass spectrometry (TOF-MS)	Uses the differences in transit time through a drift region to separate ions of different masses.
tuning	The process of optimizing the MALDI instrument's laser power and target position to obtain the best possible sensitivity and signal-to-noise ratio for a specific type of experiment.
unit resolution	Distinguishes between ions separated by 1 *m/z* unit.

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